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Stimulation of Erythropoiesis in Sublethally Irradiated Rats by a Plasma Factor.* (22152)

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It is well established that red cell production is severely depressed by low doses of whole body irradiation. Erythropoiesis can be significantly increased, however, when the animal is bled immediately before(1,2) or after(2) sublethal irradiation. A similar but less pronounced effect results from the post-irradiation administration of para-aminopropiophenone (PAPP), which produces a severe, transient methemoglobinemia. Recently evidence has been presented to indicate that in the normal animal hypoxia elicits the release of a humoral substance (hemopoietine) capable of stimulating erythropoiesis(3-6). Since both bleeding and PAPP

produce hypoxia, it might be suggested that the subsequent release of hemopoietine is responsible for the accelerated regeneration of erythropoietic tissue in the sublethally irradiated animal. If such is the case, plasma collected from donors with post-hemorrhagic anemia might be capable of stimulating erythropoiesis in the sublethally irradiated animal.

Material and methods. Female Sprague-Dawley rats weighing 135-170 g were used. In each experiment 24-30 animals were randomly assigned to 3 or 4 groups. Animals were exposed to whole body irradiation, 200 r from a Co⁶⁰ source in 4 π geometry(7) or 160-190 r from a 2.5 MEV Van de Graaff generator at an average dose rate of 50 r per minute measured with a Bendix ionization chamber known to be air equivalent at that energy. "Anemic" plasma was prepared by bleeding normal donor rats 2% of the body

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TABLE I. Effect of Plasma from Anemic Donors on Fe⁵⁹ Incorporation in Sublethally Irradiated Rat.

Exp. #	Irradiated controls	Normal plasma	Anemic plasma*		p†	—Radiation—	
			24 hr	48 hr		Source	Dose, r
1	23 ± 2.6 (7)‡	36 ± 7.8 (7)		68 ± 2.5 (5)	<.01	Co ⁶⁰	200
2	35 ± 1.7 (8)	32 ± 4.4 (7)		42 ± 2.5 (7)	<.05	2.5 MEV	160
3	31 ± 6.2 (5)	33 ± 6 (6)	51 ± 1.3 (5)	38 ± 2.9 (6)	<.01	2.5 "	160
4		39 ± 6.6 (6)	44 ± 2.7 (8)		n.s.	2.5 "	180
5		38 ± 5 (7)	53 ± 2.1 (8)		<.01	2.5 "	180
6	26 ± 4 (5)	37 ± 2.6 (6)	53 ± 4 (7)		<.01	2.5 "	190

* Plasma collected 24 or 48 hr after production of post-hemorrhagic anemia.

† p refers to probability that observed differences between groups receiving anemic and normal plasma occurred by chance. In the third experiment, 24 hr anemic plasma was compared with normal plasma. The p was determined using the one sided t test.

‡ Fe⁵⁹ values given represent % of total injected dose present in circulating red cells 48 hr after injection ± a single stand. error computed as $\frac{\text{Range}}{K_n \sqrt{n}} (10)$. Number of animals in each group is included in parentheses.

weight (approximately 30% of the blood volume) by cardiac puncture. 24 or 48 hours thereafter, blood was collected in heparin or 5% Na₂ ethylene diamine tetraacetate and the plasma separated. Control plasma was obtained from normal donors not previously bled. The plasma preparation to be tested was injected into the tail vein within 90 minutes following irradiation. An amount equivalent to 30% of the recipient's blood volume, calculated as 7% of the body weight, was injected. For studies of heat stability, anemic plasma was adjusted to pH 5.4 with 1 N HCl and heated at 100°C for 10 minutes. The precipitated protein was separated by centrifugation at 2000 G for 2 hours. The pH of the supernatant was adjusted to 7.1 with 1 N NaOH and injected as before. Fresh anemic plasma was also dialyzed at 4° for 24 hrs. 25 cc of plasma was dialyzed against 1000 cc of saline with 3 changes of the dialyzing fluid during the 24 hr period.

Rates of erythropoiesis were originally estimated by reticulocyte levels, the histologic appearance of erythropoietic tissue on the 4th post-irradiation day and iron incorporation. Since there was an excellent correlation between these measures(2), subsequent estimates of red cell production were based on Fe incorporation alone. For this determination, Fe⁵⁹ tagged normal plasma was injected on the 2nd post-irradiation day and 48 hours thereafter blood was collected by cardiac puncture for determination of Fe⁵⁹ activity.

The details of tagging of the plasma, determination of radioactivity, and the calculation of the % Fe incorporation have been given previously(2). At the completion of each experiment, the tails of the injected rats were checked for radioactivity. The rare animals in which more than 5% of the injected Fe⁵⁹ was present in the tail, indicating substantial extravascular injection, were excluded from the series. Rats with pneumonia, which occurs in 2 to 5% of animals in our colony, were also excluded after verification of the diagnosis by autopsy.

Results. The average Fe⁵⁹ incorporation in 48 hours by normal non-irradiated animals was 65%, with individual animals varying from 50-80%. The average Fe⁵⁹ incorporation for animals receiving 160-200 r of whole body irradiation was 25% (Co⁶⁰ source) and 30% (2.5 MEV source) with a range of 10-45%. The injection of normal plasma following irradiation produced a variable response. In 3 of 4 experiments, where animals receiving normal plasma were directly compared with irradiated controls (Table I), the Fe⁵⁹ incorporation of those receiving normal plasma was greater than in the irradiated controls. In only one of these, however, was the difference significant (Exp. 6, Table I, $p < .02$). The variability within each group receiving normal plasma was greater than that observed with control animals or those receiving anemic plasma (Table I).

The injection of plasma from anemic don-

TABLE II. Studies on Stability of Erythropoietic Factor in Anemic Plasma.

Normal plasma	Anemic plasma*				
	Fresh	Stored		Dialyzed	Heated
		4°	-20°		
38 ± 5 (7)†	53 ± 2.7 (8)	44 ± 5 (7)	51 ± 6 (5)		
39 ± 6.6 (7)	44 ± 2.7 (8)			44 ± 2.5 (5)	34 ± 5.7 (4)

* Plasma collected 24 hr after production of post-hemorrhagic anemia.

† Fe⁵⁹ values given represent % of total injected dose present in circulating red cells 48 hr after injection ± a single stand. error computed as $\frac{\text{Range}}{K_n \sqrt{n}} (10)$. Number of animals in each group is included in parentheses.

ors into irradiated animals produced a significant increase in the rate of erythropoiesis as measured by Fe⁵⁹ incorporation (Table I). In all experiments to date the average Fe⁵⁹ incorporation values were greater in the animals injected with anemic plasma than in those receiving normal plasma or in untreated irradiated controls. The difference in the means of groups receiving anemic and normal plasma varied from 5-32% (Table I). In 4 of 6 experiments the differences in mean Fe⁵⁹ incorporation between animals receiving anemic and normal plasma were significant at a 0.01 level, and in one at a 0.05 level (Table I). In the remaining experiment, the mean for anemic plasma was greater than for normal plasma, but the difference was not statistically significant ($p > 0.05$). There was marked variation in the response between experiments but the variability within a given experimental group was relatively small (S.E. 1.3-4).

In one experiment anemic plasma collected 24 hours after the donor animals were bled produced a greater response than that collected 48 hours after bleeding (Table I). The factor present in anemic plasma retained its activity during storage for 3 weeks at -20°C (Table II). The supernatant obtained after heating at 100°C at pH 5.4 for 10 minutes was inactive (Table II). There was no reduction in the activity of anemic plasma following saline dialysis at 4°C for 24 hours (Table II).

Discussion. It has been suggested that the modification by acute blood loss of the usual irradiation injury to erythropoiesis is due to the elaboration of hemopoietine which accelerates the regeneration of erythropoietic tissue. This interpretation has been supported

by the observation that plasma collected from anemic donors, when injected into rats immediately following sublethal irradiation, stimulated erythropoiesis as evidenced by an increase in mean Fe⁵⁹ incorporation of 5-30% above that of animals receiving normal plasma. As might be expected, the magnitude of response in animals receiving anemic plasma was not as great as had been noted previously in animals with acute blood loss following irradiation. Although there was a considerable variation in the degree of response between experiments, all groups receiving anemic plasma had a mean Fe⁵⁹ incorporation greater than that observed following the administration of normal plasma. In 4 experiments the differences were significant at the 0.01 level, in 1 at the 0.05 level and in 1 there was no significant difference ($p > .05$). The response of the sublethally irradiated animals to anemic plasma, therefore, appears to be highly significant. The stability of the factor present in anemic plasma was similar to that reported by Erslev (8).

The variability of response to anemic plasma between experiments may be accounted for in part by differences in the plasma content of hemopoietine. The marked difference between the experiment in which a Co⁶⁰ source was used and those with the 2.5 MEV source, suggests that irradiation factors may account for some of the variability.

Normal plasma produced an increase in Fe⁵⁹ incorporation in 3 of 4 experiments where there was a direct comparison with irradiated controls but in only one was the difference statistically significant. This effect may have resulted from the presence of small amounts of the erythropoietic substance in

normal plasma or from a transient dilution anemia. The marked variability within groups receiving pooled normal plasma (S.E. of the mean 2.6-7.8) is thought to result from variations in responsiveness of individual animals to borderline amounts of hemopoietine.

It is of interest that only a single injection of anemic plasma is required to demonstrate erythropoietic activity in the sublethally irradiated animals, whereas multiple injections are required in normal animals(4,6). It has previously been observed that a single injection of PAPP, which fails to produce a significant alteration of erythropoiesis in the normal rat(9), will increase red cell production when administered to rats following a sublethal dose of irradiation (120-200 r from a 3 MEV source, 200 r from a Co⁶⁰ source)(2). These observations suggest that immediately following irradiation an animal is more sensitive to erythropoietic stimuli than normally, provided that the dose of irradiation is not sufficient to destroy the majority of the erythropoietic tissue. It is tempting to suggest that the increased sensitivity of the irradiated animal results from the destruction of an inhibitor thus altering the normal balance between inhibitors and stimulators of erythropoiesis. The sublethally irradiated rat may serve as a useful animal for the further investigation of the regulation of erythropoiesis, since the method employed requires but a single treatment, allows a satisfactory estimate of red cell production by the Fe⁵⁹ incorporation technic and requires only 5 days for an experiment. At present, the method requires

the use of 5-7 animals per group and the inclusion of controls. The variability of response may be in part a reflection of the plasma content of hemopoietine rather than the lack of sensitivity of the test.

Summary. Injection of plasma from anemic donors into rats immediately following exposure to sublethal doses of X-rays produced a statistically significant increase in erythropoiesis as measured by Fe⁵⁹ incorporation. The factor present in anemic plasma was non-dialyzable and was not present in the supernatant obtained after precipitation of protein by heating. Normal plasma produced a variable response. Use of sublethally irradiated rat as a test animal in the study of regulation of erythropoiesis is suggested.

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Absorption and Elimination of Carbon¹⁴ Labelled Nicarbazine by Chickens. (22153)

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Nicarbazin, an equimolecular complex of 4, 4'-dinitrocarbanilide (DNC) and 2-hydroxy-4, 6-dimethylpyrimidine (HDP), is effective for prevention of coccidiosis in chickens(1). This complex is split into its com-

ponents in the animal, and the HDP portion rapidly eliminated. By use of spectrophotometric methods, both moieties of nicarbazine have been detected in tissues of chickens receiving the drug(2). However, 2 days after

TABLE I. DNC in Chicken Tissues following the Oral Administration of Nicarbazine.

Tissue	Time after final dose (hr)	DNC and metabolites, γ /g tissue calculated as DNC	DNC (colorimetric), γ /g tissue	Ratio: Radioactive/Colorimetric
Plasma	3	4.4	3.8	1.2
	24	7.2	4.9	1.5
Liver	3	41.8	32.0	1.3
	24	67.8	33.0	2.0
Thigh muscle	3	9.7	4.8	2.0
	24	11.3	4.6	2.5
Breast muscle	3	6.8	4.7	1.4
	24	9.9	2.9	3.5

withdrawal of nicarbazine from the diet, neither DNC nor HDP could be detected in the tissues by chemical methods.

There was the possibility, however, that metabolites of DNC undetected by the chemical means used might remain in the tissues. Therefore, nicarbazine labelled with C^{14} in the benzene ring of DNC was prepared and fed to chickens. The data show that after discontinuance of treatment, both DNC and its metabolites are rapidly eliminated from the tissues.

Exp. 1. Two 200 g chickens were given oral doses of labelled nicarbazine (activity, $0.05 \mu\text{C}/\text{mg}$)* suspended in 2% methyl cellulose solution. Each bird received 3 doses of 126 mg at 3 hour intervals. One chicken was sacrificed 3 hours, the other 24 hours after the final dose. Aliquots of aqueous tissue homogenates were dried at room temperature in stainless steel planchets and counted in a Q-gas flow counter. Counts were corrected for background and for self-absorption. Counting was done for sufficient time to assure statistical accuracy of 1% to 4%, depending upon activity of the sample. Colorimetric estimation of DNC in the homogenates was by the method previously described (2).

Results. The ratio of apparent DNC, determined by radioactivity measurements, to that found by the chemical method ranged from 1.2 to 3.5 (Table I). It is evident that DNC metabolites were present in the tissues. Although tissue samples taken 3 and 24 hours

after dosing were from different chicks, the data suggested that during the 24-hour period, some accumulation of DNC metabolites had occurred.

Exp. 2. Groups of 12 chickens (one-week-old New Hampshire Reds, weighing 70-84 g) were fed diets containing 0.015% or 0.06% labelled nicarbazine. Radioactivity measurements were made on blood taken daily from 6 chickens of each group, each group of 6 being bled on alternate days. On the seventh day, 3 birds from each group were sacrificed. The remaining chicks were placed on stock rations, and 3 from each group sacrificed 1, 2

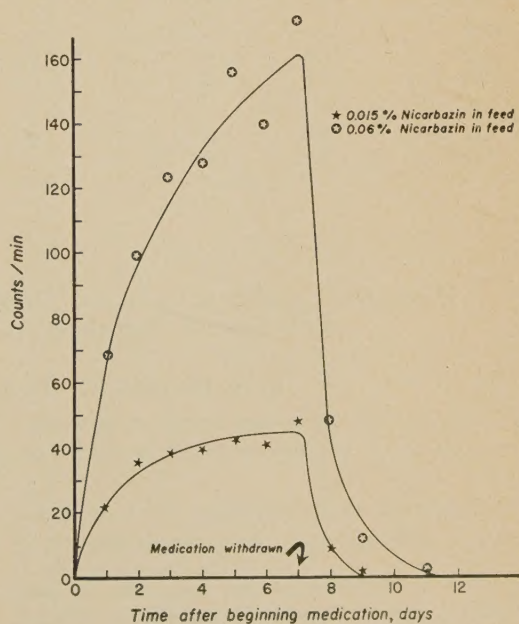


FIG. 1. Radioactivity in blood of chickens during and after medication with C^{14} -labelled nicarbazine.

* We are indebted to Dr. Elbert Harris, Chemical Division, Merck & Co., Inc., who made the material available to us.

TABLE II. DNC in Chicken Tissues after the Feeding of Nicarbazine in the Diet.

Days after medication discontinued	0	1	2	4
Tissue	DNC, γ /g wet tissue or γ /ml blood plasma			
0.015% nicarbazine in feed				
Thigh muscle				
Radio.	1.8	.5	.35	.32
Chem.	4.1	.0	.0	—
Breast muscle				
Radio.	2.1	.6	.0	.03
Chem.	1.7	.0	.0	—
Kidney				
Radio.	9.0	.2	.05	.0
Chem.	5.5	.0	.0	—
Liver				
Radio.	14.5	3.8	.54	.01
Chem.	8.0	1.0	.0	—
Blood				
Radio.	2.7	.5	.03	.05
Chem.	1.8	.1	.0	—
0.06% nicarbazine in feed				
Thigh muscle				
Radio.	12.1	3.3	2.0	.19
Chem.	14.3	1.9	.4	—
Breast muscle				
Radio.	7.4	2.4	.4	.24
Chem.	7.2	2.0	.0	—
Kidney				
Radio.	31.5	1.1	.17	.0
Chem.	25.7	2.9	.0	—
Liver				
Radio.	25.7	11.3	3.6	.24
Chem.	15.6	9.6	.4	—
Blood				
Radio.	9.6	2.8	.75	.14
Chem.	7.0	1.4	.1	—

and 4 days later. Tissues and blood were assayed as in Exp. 1.

Results. Radioactivity in blood of chickens fed 0.015% nicarbazine (Fig. 1) approached a plateau in the 7-day feeding period. The slope of the curve relating radioactivity to time for birds fed 0.06% nicarbazine, decreased with time. If a longer feeding period had been used, it is probable that this curve also would have approached a plateau. Withdrawal of medication was followed by a precipitous fall in radioactivity to zero in 2 to 4 days.

Radioactive counts and chemical analyses of the tissues (Table II) agreed in demonstrating that two days after withdrawal of 0.015% medication, and 4 days after withdrawal of 0.06% medication, only traces of DNC or its metabolites were present in the tissues.

Summary. Tissues of chickens fed diets containing 0.015% and 0.06% radioactive nicarbazine for 7 days contained DNC and minimal quantities of its metabolites. Two and four days, respectively, after withdrawal of 0.015% and 0.06% medication, only traces of DNC and its metabolites remained in the tissues.

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Hormone Content of Anterior Pituitary of Monkey (*Macaca mulatta*)* with Special Reference to Gonadotrophins. (22154)

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Pituitaries from monkeys, of both sexes and of different ages, have been assayed in

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hypophysectomized rats for their content of gonadotrophic hormones. These studies were undertaken to determine any peculiarities either in quantity or proportion of the gonadotrophic hormones, preliminary to treatment of macaques with preparations from homolo-

gous anterior pituitaries. Estimates of levels of other pituitary trophic hormones were made concurrently.

Methods. Pituitaries were removed from adult female monkeys at representative stages of the menstrual cycle, and from adult and immature males, for assay of hormone content of individual glands.[†] Pituitaries were also collected from large numbers of males and females of variable ages, and pooled for assay and future chemical fractionation. The anterior lobes were dissected free from dura and from posterior and intermediate lobes. Anterior pituitaries from individual animals were frozen at once and lyophilized; pooled anterior lobes were frozen within 2 hours after sacrifice. Wet and dry weights of individually collected anterior pituitaries were determined, and average weight in pooled lots could be calculated from the collective wet or dry weight and the number of glands. Assays were performed in immature female rats, hypophysectomized at 26-28 days of age, beginning 6 to 8 days after operation. Groups of 3 rats were used for each dose level. No data from incompletely hypophysectomized animals have been included. The postoperative period of one week used in most experiments was selected as fulfilling a condition defined in assay of gonadotrophins, follicle stimulating hormone (FSH) and interstitial cell stimulating hormone (ICSH). The approximate titer of the other trophic hormones of the pituitary, growth hormone (GH), adrenocorticotrophic hormone (ACTH), and thyrotrophic hormone

[†] Pituitaries of adult female monkeys in known stages of cycle were obtained from the Yale Obstetrics Colony. Pituitaries of immature male monkeys used to make implants of individual glands were obtained from the Virus Laboratory, University of California, through the courtesy of Dr. Mary McClain. The large numbers of glands which were pooled, frozen and dried, were obtained from the Cutter Laboratories through the kindness of Dr. Walter E. Ward, Dr. Donald Trotter and Mr. Donald H. Wonder. Pituitaries from estrinized monkeys (450 μ g estradiol benzoate over a period of one week previous to autopsy) were obtained through the courtesy of Dr. H. R. Catchpole, University of Illinois School of Medicine.

TABLE I. Hormone Assay of Anterior Pituitaries of the Monkey. MED expressed as total dose in dry weight (mg).

Sex	Donors Age Cycle day	Wt, kg	Test conditions	Wt (mg)		Assay in hypophysectomized rats (mg)				
				anterior lobe	Dry	FSH	ICSH	ACTH	TSH	GH
Females	Adult	5.4	Subcut.: FSH, ACTH, TSH	59	13.2	1.0	.25	1.5	1.0	.25
	1	5.0	IP: ICSH, GH	58	12.5	1.5	.75	2.5	1.0	.08
	9	4.2	Daily, 3 da; 72 hr	43	9.8	.25	.1	>1.5	.18	.08
	9	6.8		48	11.9	.5	.15	1.0	>1.5	.05
	11	6.4		57	12.8	1.0	.1	>1.5	>1.5	.08
	15	4.5		46	10.0	2.5	.25	2.0	.25	.30
	22	4.3		42	9.6	1.5	.5	1.6	.3	.08
	Immature (estrin)	2.1	As above	6.7	6.7	>1.0	>1.0	>1.0	>1.0	.10
		2.1		6.3	6.3	>1.0	>1.0	.5	.5	.10
	Adult	8.2	As above	36	9.7	.75	.3	>2.0	1.5	.08
Males	Immature	3.0	One implant; 96 hr	24	6.0*	>3.0	>3.0	1.5	<1.5	<1.5
		3.4	<i>Idem</i>	20	5.0*	>2.5	>2.5	>2.5	1.2	<1.2
♂ & ♀ pooled	Variable ages		Subcut. 4 da; 96 hr	13.9	3.2	3.2	3.2	6.4	.8	<.8

* Implanted fresh; approximate dry wt derived by factor of 4.

TABLE II. Hormone Content of Anterior Pituitaries of the Monkey.

Sex	Donors		Total units per anterior lobe				
	Age	Cycle day	FSH	ICSH	ACTH	TSH	GH
Females	Adult	1	13	53	9	13	53
		1	8	17	5	13	150
		9	40	100	<7	54	120
		9	24	80	12	<8	240
		11	13	130	<9	<9	160
		15	4	40	5	40	30
		22	6	19	6	30	120
	Immature (estrin)		<7	<7	<7	<7	67
			<7	<7	13	13	63
Males	Adult		13	32	<5	5	120
	Immature		<2	<2	4	>4	>4
			<2	<2	≤2	4	>5
♂ & ♀ pooled	Variable ages		≥1	≥1	.5	>4	4

(TSH), could also be determined even though the postoperative period was too short to allow for an entirely satisfactory state of regression of the target organs.

Results. Potency of anterior pituitaries from various types of monkeys is expressed in Table I as the minimal effective dose (MED) or unit value for each of the 5 principal trophic hormones.[†] Total hormone content expressed in units per anterior lobe is given in Table II.

Pituitaries of Normal Adult Females. An-

[†] MED for *follicle stimulating hormone* is defined as the minimal total dose which will cause microscopically detectable growth of ovarian follicles beyond control level, specifically to the stage of beginning antrum formation. The MED for *interstitial cell stimulating hormone* is the total dose which will cause microscopic evidence of beginning repair of the "deficient" type of interstitial tissue, including enlargement of nuclei, lighter nuclear stain and reappearance of a rim of eosinophilic cytoplasm. The MED for *thyrotrophic hormone* is the total dose which causes increase in height of epithelium, from squamous to low cuboidal, with beginning vacuolation of colloid. MED for *growth hormone* is total dose which will cause an increase of 30 to 50 micra in width of uncalcified portion of proximal epiphyseal cartilage of tibia stained with silver nitrate. MED for *adrenocorticotrophic hormone* is total dose which causes beginning repair of adrenal cortex as seen in frozen sections stained with Sudan black; the criteria include replacement of coarse by fine lipid droplets, infiltration of lipid into the subglomerular lipid-free zone, increase in cell size and width of cortex, and regeneration of the zona reticularis.

terior lobes from adult female monkeys sacrificed at known stages of menstrual cycle, were pulverized, suspended in saline and injected daily for 3 days. The MED for FSH ranged from 2.5 to 0.25 mg dry weight of gland; thus the total hormone per anterior lobe varied between 4 and 40 units. The titer of FSH was highest on ninth day of cycle, the MED being 0.5 to 0.25 mg; 24 to 40 units were therefore present per gland. In early and late periods of the cycle the MED varied from 2.5 to 1.0 mg, with 4 to 13 units present per gland.

Unitage of ICSH was higher than that of FSH. The MED varied from 0.75 to 0.1 mg and total content ranged from 17 to 130 units per gland. Potency was highest between days 9 and 11, MED being at this time about 0.1 mg with 80 to 130 units present per gland. The content of ICSH increased relatively more than that of FSH during middle of the cycle, so that the ratio of ICSH to FSH increased above its usual value of 3 to 1, and reached a value of 10 to 1 on days 11 and 15. Evidence from the response of hypophysectomized recipients is not conclusive that monkey pituitary at any specific period in the cycle contained the optimum ratio for corpus luteum formation. It is to be noted, however, that corpus luteum formation with ovulation was induced by a total dose of 1.5 mg of pituitary tissue taken during first half of menstrual cycle (days 1, 9 and 11), whereas even higher doses of pituitaries, removed during

the last half of the cycle (days 15 and 22), resulted only in minimal follicular stimulation in hypophysectomized rats. Growth hormone was present in considerable unitage in the adult female pituitaries, demonstrable at 0.3 to 0.05 mg total dose. There was no significant change in minimal effective dose or in total pituitary content during the cycle, more than 100 units per pituitary usually being demonstrable. The adrenocorticotrophic hormone content was consistently low, the hormone frequently detectable by change in lipid pattern in the adrenal of the recipient only at the highest levels tested, 2.5 or 1.5 mg. At most, 5 to 10 units were present per anterior pituitary. The minimal effective dose for thyrotrophic hormone was usually between 1.0 and 0.2 mg, total unitage ranging between 10 to 50 units per pituitary, with no cyclic pattern.

Pituitary of adult male. The anterior pituitary from an adult male macaque was assayed as above. The weight of anterior pituitary was about the same as the smallest from the normal adult females. Unitages of various hormones were roughly within the same range as in normal adult female pituitaries, though the content of FSH did not equal that reached in the female pituitary in the middle of the cycle. The total amount of ICSH present per anterior pituitary was within the lower range found in the normal female. The ratio between total content of ICSH and FSH (2 to 1) was as low as any encountered in the female pituitary, either at beginning or end of the cycle. Ovulation and corpus luteum formation in hypophysectomized rats resulted from total doses of 2 mg. The content of GH was comparable to that in the adult female, that of TSH and ACTH was even lower than in normal females.

Pituitaries of immature males. Assays of pituitaries of immature males were not strictly comparable to those of the normal adult male pituitary just cited, inasmuch as repeated injections were not given but fresh anterior lobe tissue was implanted. The content of all hormones, notably that of gonadotrophic hormones, appeared to be lower than in adult pituitaries.

Pooled pituitaries of monkeys. These pituitaries were collected from male and female monkeys of unknown age, though predominantly from young animals. The frozen-dried pituitaries were injected in saline suspension daily for 4 days. The hormone content per pituitary was lower than that in the individual adult female or male pituitaries but was within the same range as in individual immature male pituitaries.

Pituitaries of estrinized immature females. Pituitaries removed from 2 females which had received estradiol benzoate were lyophilized and assayed individually as above. The pituitaries were small. Neither FSH nor ICSH could be detected, even after injection of much higher doses than were required for normal adult female or for pooled pituitaries. The content of ACTH, GH and TSH was in the low normal range.

Discussion. Very few accounts have been found in the literature pertaining to the content of gonadotrophic or other trophic hormones in monkey pituitary. Salhanick, Hisaw and Zarrow(1) examined gonadotrophic potency of pituitaries of castrate macaques and of castrates treated with estradiol. Gonadotrophic potency of the castrate pituitary was high and was depleted by estrogen therapy; no value was given for hormone content in the normal animal. As stated in the present paper, gonadotrophins could not be detected, even at high doses, after estradiol treatment of immature females.

The hormone content of human pituitaries has received more attention. Some studies which should be mentioned are those of Witschi and Riley(2), Bruner(3), Bahn, Lorenz, Bennett and Albert(4-7), Leatham(8), and Burt and Velardo(9). Assays conducted by Witschi and Riley, and by Bahn *et al.*, have probably been the most comprehensive, including assays of pituitaries of normal adult men and women, of postmenopausal and pregnant women, of old men and of children. Those of Bahn and collaborators are particularly significant for comparison with our results for monkey pituitaries, because the same type of assay animal (the hypophysectomized rat) and similar morphological criteria were

used in establishing unitage. Furthermore, these authors assayed anterior lobe tissue free of posterior lobe so that their estimates of hormone content can be compared directly with those given here. A total dose of 3 mg of dry anterior pituitary tissue from women was the minimal amount necessary for detection of either FSH or ICSH. In the adult female monkey the minimal amount needed for demonstration of FSH ranged from 2.5 to 0.25 mg, and of ICSH from 0.75 to 0.1 mg, both varying with the menstrual cycle. The ratio between minimal effective doses for ICSH and FSH was 2 or 3 to 1 at beginning and end of the cycle and rose to 10 to 1 on days 11 and 15, whereas a ratio of unity was reported for the human pituitary. Bahn *et al.* found that the MED for gonadotrophic hormones was the same in men as in women, the ratio of ICSH to FSH again being unity. The values given here for minimal effective doses of adult male monkey pituitary were lower; ICSH 0.3 mg, FSH 0.75 mg, and the ratio was 2.5 to 1. Both Bahn *et al.*, (6) and Witschi and Riley (2) have found a low gonadotrophic content in pituitaries of children, the content being 1/5 to 1/10 that in adult pituitaries. The gonadotrophic hormone content of pituitaries of immature monkeys recorded here was correspondingly low.

The assays of monkey pituitaries were made to determine whether their gonadotrophins were peculiar either qualitatively or quantitatively, in the hope that such data might guide efforts to induce sexual maturation and ovulation in monkeys. All pituitary preparations hitherto injected in such experiments have been from heterologous pituitaries (chiefly from pig and sheep). The gonadotrophic potency of rhesus pituitaries was higher than that of sheep pituitaries. The MED for both FSH and for ICSH in adult female monkey pituitaries at the time of highest hormone content was 1/10 that for sheep pituitary (10). The two gonadotrophic hormones were present, however, in the same proportion in sheep and monkey pituitaries. In acetone dried sheep pituitaries the MED for ICSH was 1 mg, for FSH 2.5 mg; in lyophilized monkey anterior lobe, the MED for

ICSH was 0.1 mg, for FSH 0.25 mg.

In the monkey anterior pituitary the content of FSH was highest on 9th day of menstrual cycle, compatible with the supposed preovulatory spurt of follicular growth at about this time. The content of ICSH was highest in preovulatory and ovulatory period, consistent with the synergistic action between ICSH and FSH, and its probable role in final follicular enlargement, and also with the importance of ICSH in corpus luteum formation. The ratio of ICSH to FSH was highest in ovulatory and early postovulatory period. However, pituitaries from female monkeys in the first half of the cycle, as well as those from the adult male, caused ovulation in hypophysectomized recipients, even though in both types of pituitaries the ratio of ICSH to FSH was low. Failure of pituitaries from female monkeys in the last half of the cycle to induce ovulation must have been due to low unitage, particularly of FSH.

Summary. Individual anterior pituitaries as well as pooled glands of *Macaca mulatta* have been assayed in hypophysectomized immature female rats for their content of trophic hormones, particularly gonadotrophic hormones. 1. In the adult female macaque, the content of follicle stimulating hormone was highest between the ninth and eleventh days of the cycle. The content of interstitial cell stimulating hormone was also maximum at this time of cycle. The ratio of ICSH to FSH was highest (10 to 1) on days 11 and 15. 2. In adult male anterior pituitary the total content of ICSH and FSH and the ratio between these hormones was approximately the same as in the adult female pituitaries at beginning and end of menstrual cycle. The gonadotrophic hormone content of pituitaries of immature animals was lower than in adults. 3. Other trophic hormones in the adult female pituitary, thyrotrophic, adrenocorticotrophic and growth hormones, did not change in amount during the menstrual cycle. In adult male, as well as in the female pituitary, thyrotrophic and adrenocorticotrophic hormones were present in low concentration, but growth hormone was present in high unitage.

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Determination of Evans Blue Dye in Blood and Tissues.* (22155)

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To our knowledge there is only one report in the literature of a method for determining Evans blue dye in whole blood(1) and only 3 for determining the dye in tissues(2-6). The present paper describes a new procedure for its estimation in tissues, of which blood may be considered a special case. In addition, by employing this method, data have been obtained on the fate of the dye for up to 140 hours after its injection into the rat.

Method. Description of method. The method is based upon 3 fundamental operations: (1) homogenization of tissue in a concentrated solution of urea, (2) splitting of the Evans blue-protein complex and partial precipitation of chromogens with acetone, and (3) complete precipitation of chromogens by addition of Somogyi reagents. Details of the method as employed for tissues are as follows: (1) A 5-g sample of the dye-containing tissue to be analyzed and 2 additional 5-g dye-free samples of similar tissue obtained from another animal were weighed. To one of the latter was added a known amount of dye in approximately the amount to be found in the unknown tissue sample. The 3 samples (tissue plus unknown dye, tissue plus known dye, tissue without dye) were minced. (2) Concentrated urea solution (equal weights of A.R. urea and distilled water) was added to each tissue sample until the total volume was

100 ml and the mixture was homogenized in a Waring Blendor for 10 minutes. The homogenate was poured into a flask in a cold tap water bath and allowed to cool for 5 minutes. (3) Two hundred ml of A.R. acetone added to each sample and each was shaken briefly. Ten ml of Somogyi reagent I (10% zinc sulfate • 7H₂O) was added to each sample which was then mixed well by shaking. After further addition of 10 ml of Somogyi reagent II (0.5 N NaOH) to samples, the latter were mechanically shaken for 10 minutes and allowed to stand 15 minutes. (The 2 Somogyi reagents should be standardized by titration against each other to the end point of phenolphthalein). (4) The samples were filtered through Whatman No. 42 paper. Optical density of filtrates from the 2 dye-containing samples was determined at 620 mμ in colorimeter which has a 20 cm cell length. The tube containing dye-free tissue was used for the I₀ setting. **Procedure for analysis of blood.** The method was applied to blood as follows: (1) one-half to 2 ml of whole blood was taken and 0.9% NaCl was added where necessary to bring the final volume to 2 ml. Two ml of the urea solution used for tissue analyses was added followed by 8 ml of A.R. acetone and 0.5 ml each of the 2 Somogyi reagents. The samples were shaken between each addition. (2) After centrifugation at high speed for 15 minutes the supernatant liquid was taken for colorimetry at 620 mμ.

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TABLE I. Recovery of Added Evans Blue from Dog Tissues.

Tube No.	Contains	Optical density	% yield
I ₀	Water only	.0	0
1	All reagents (a check on water as I ₀)	.0	0
2	Dye + reagents (except Somogyi reagents)	.218	100
3	Heart muscle, no dye	.002	1
4	" " + "	.222	102
5	Intestine, no "	.0	0
6	" " + "	.218	100
7	Brain, no "	.002	1
8	" " + "	.215	98.5
9	Liver, no "	-.002	-1
10	" " + "	.215	98.5
11	Kidney, no "	.002	1
12	" " + "	.218	100

The standard solution was made with known amounts of dye added to an amount of whole blood equal to that taken for analysis of the unknown. The solution used for the I₀ setting was made from dye-free blood. With less than 0.5 ml of blood the optical density is a function of amount of blood because of the effect of blood colloids and salts on absorption of light by the dye. *Fate of dye in rats.* 1. While under sodium pentobarbital (Nembutal) anesthesia each of 8 fasted rats was injected with 0.25 ml of 5% Evans blue dye into the exposed right jugular vein. Twenty minutes later was taken as zero time, and concentration of dye in a 0.2-ml sample of tail blood at this time was taken as 100%. The animals recovered from the anesthetic 4 to 8 hours later. The animals sacrificed within 16 hours were placed in cages without food but with water. The animals sacrificed after 16 hours were placed in cages with food and water but were taken off food for 12 hours before they were sacrificed. At various time intervals up to 140 hours individual rats were stunned by blow on head and 0.2 ml blood was taken from the still beating heart. Blood and whole rat carcass were analyzed for dye by methods previously described using a 6% correction for weight loss of rat tissues caused by evaporation of water during grinding. Excreta were included in the carcass analysis only in the case of rats sacrificed before recovery from anesthesia. To judge from color of urine and feces, their dye content was relatively trivial.

Results. Recovery of dye from various tissues. Two experiments were run, each on a set of 5 different tissues obtained from dogs. In the first experiment 0.0376 mg of Evans blue was added per gram of experimental tissue and a colorimeter equipped with a 20 cm cuvette was used. In the second experiment 0.10 mg of dye was added per gram of tissue, and the Coleman Spectrophotometer equipped with a 1 cm cuvette was employed. Since data from both experiments were essentially the same, only those from the second experiment (0.1 mg dye per g of tissue) are given (Table I). Recoveries of added dye ranged from 98-102%. Blood was added to dye-containing tissue samples to determine the contribution of blood pigments to non-dye color in the final solution. The results (Table II) indicate that added blood up to 50% of weight of the tissue sample does not result in a positive error. More blood than this yields erroneously high dye recoveries.

Recovery of injected dye by analysis of the whole animal. Evans blue was administered intravenously to each of 14 rats and the whole

TABLE II. Effect of Added Blood on Dye Recovery from Tissues.

Sample	g of tissue	ml of blood added	% yield
Control	0	0	100.0
1	5	0	101.5
2	5	2.5	100.0
3	5	5.0	106.5
4	5	7.5	111.9
5	5	10.0	117.5

TABLE III. Assay of Whole Rat for Evans Blue Content.

Sample	Optical density	% yield on basis of raw data	% yield corrected for wt loss during grinding
Rat	.444	102.8	98.8
Control	.432	100.0	—

animal was taken for analysis. The following is a typical protocol: A rat weighing 409 g was fasted overnight and anesthetized with sodium pentobarbital (Nembutal), 35 mg/kg given intraperitoneally. One ml of 1.0% Evans blue dye was injected into the exposed jugular vein. It was necessary to fast the animals because fox chow in the gastrointestinal tract contributed errors of 7 to 28% due to a green color which is extractable from the chow and which appears in the final solution used for colorimetry. One ml of the same 1% dye solution was added to 409 ml of water to serve as a standard. The rat was killed by a blow on the head following which it was quartered and ground in a meat grinder 6 times. The tissue mass was blended with spatulas between the several grindings. After grinding, the tissue weighed 393.6 g, a loss of 15 g from evaporation of water. The final result was corrected for this weight loss. Five g of the tissue were taken for analysis. A second rat which received no dye was ground in the same manner and 5 g of its tissues were added to 5 ml of the standard solution. Another 5 g of tissue from the second rat was employed for preparation of the solution used for the I_0

TABLE IV. Recovery of Added Evans Blue from Whole Rats.

Rat No.	% yield (corrected)*
1	103.2
2	97.7
3	97.1
4	97.5
5	102.5
6	101.1
7	101.2
8	103.2
9	101.0
10	97.1
11	99.5
12	98.6
13	102.9
Rat from Table II	98.8

* For wt loss during grinding.

setting. All samples were homogenized in concentrated urea solution and treated with acetone and the Somogyi reagents as described above. Colorimetry was done at 620 mμ using a colorimeter equipped with a 20 cm cuvette. The results are given in Table III. In Table IV are given the results for the remaining 13 rats. In this series the method was accurate within 3% of the true value. In preliminary trials, proportions of urea solution and tissues, amounts of the 2 Somogyi reagents and the amount of acetone were varied independently. The method as specified is based upon optimal reagent amounts as established in these trials.[†] It was necessary to add dye-free tissue to the standard dye sample to prevent the shift of dye color from dark blue to light purple, as may occur in acetone-water solutions in the presence of Somogyi reagents.

Fate of injected dye in rats. The data are presented in Fig. 1, in which the lines were fitted to the plotted points by visual inspection. Because of this and because each point for a given time interval was obtained from a single animal, only the general features of the curves may be considered as meaningful. Furthermore, the points for percentage of dye in the body which is extravascular have been calculated on the assumption that blood volume has remained constant, *i.e.*, that the amount of intravascular dye is directly proportional to blood dye concentration. For large changes in blood dye concentration, errors introduced by this assumption may be considered of secondary importance. Proceeding on the basis of this assumption, one finds that 40 hours after its administration, about 90% of the dye has left the blood but approximately 83% is still in the whole carcass. Even after 140 hours, when about 92% has left the blood, some 67% can be still recovered from the carcass. Disappearance of the dye from the body is most rapid 10-24 hours after injection, and is attributed mainly to decolorization within the body rather than loss via the excreta. Thus most of the dye which presumably disappears from blood in colored form can be recovered from the whole animal even at 140 hours. This finding might be ex-

[†] Details of these trials are available elsewhere(8).

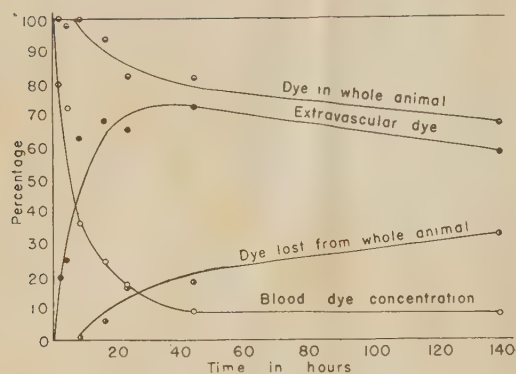


FIG. 1.

plained by the presence in blood of material which yields color by carcass analysis but not by blood analysis. This is highly improbable and, moreover, no blue color was observed in Somogyi precipitates of blood. The conclusion appears justified that the dye in the carcass not accounted for by blood analysis is in the extravascular spaces.

Discussion. Sellers *et al.* (7) have reported serum Evans blue/serum protein concentration ratio as a function of time up to 70 hours after intravenous injection into rats of 20 mg of Evans blue. They make no mention of employing anesthesia except for terminal sampling. If serum protein concentration is assumed to remain constant, their curve can be considered a blood dye concentration curve, and in fact their curve is almost identical with that for blood in Fig. 1. This similarity suggests that the present findings were not greatly influenced by the ether anesthesia.

Summary. 1. A method is described for determination of Evans blue dye in various animal tissues. In brief it consists of (1) homogenization of tissue in a concentrated solution of urea, (2) splitting of the Evans blue-protein complex and partial precipitation of protein and chromogens with acetone, and (3) complete precipitation of chromogens by addition of Somogyi reagents. 2. The method has been employed to follow the fate of the dye after intravenous injection into rats. After such administration of 12.5 mg of Evans blue, about 90% of the injected dye apparently left the blood but only about 15% could not be recovered from the body in 40 hours. At 140 hours after injection about 67% of the injected dye could still be recovered from the body.

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Plasma Prothrombin Time and Hematocrit Values of Blood of Dairy Cattle.* (22156)

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Hemostasis is a complicated mechanism of blood coagulation composed of many coordinated reactions (1-3). According to Quick (2), coagulation of blood occurs in 3 stages,

none of which are complete when considered individually. These stages must be combined in a definite arrangement before a complete explanation of hemostatic mechanism can be offered.

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Determination of plasma prothrombin time

has become essential in diagnosing, treating, and studying hemorrhagic diseases in humans (4). Since prothrombin level of blood in animals is decreased by dicumarol present in damaged sweet clover (5), in avitaminosis-K (6), and in other hemorrhagic diseases (7,8), normal prothrombin time is of considerable value in veterinary medicine. Pritchard *et al.* (9) found clotting time of blood to be lengthened when soybeans extracted with trichloroethylene were fed to cattle, but plasma prothrombin time was not lengthened appreciably. A direct correlation between plasma prothrombin time and blood clotting time was not found. This emphasizes the need for both plasma prothrombin time and blood clotting time in research with hemorrhagic diseases. A normal prothrombin time (adequate prothrombin in blood) is possible with prolonged clotting time. In this case it appears that deficiency of any one of the items (labile factor, fibrinogen, calcium, or thromboplastinogen) described by Quick (2) in blood coagulation may be present. On the other hand, prothrombin time may be prolonged with normal coagulation time. Normally excess of prothrombin is present in plasma; therefore hypoprothrombinemia must be marked before coagulation time is lengthened. Prothrombin time of plasma of sheep has been reported (10). There is, however, little information available on other ruminants. Prothrombin time of cattle is herein reported.

Methods. The first phase of this investigation was made possible through cooperation of several dairy farmers. Cows had been fed a standard dairy ration in addition to pasture grazing. Sweet clover pasture or clover hay had not been fed the past year. In the second phase, calves from another experiment (11) were divided into 3 groups. Group I calves were fed a standard calf-starter mix (basal); Group II calves were fed basal ration plus 5 trace minerals (Fe, Cu, Co, Mn, and Zn); and Group III calves were fed basal ration, trace minerals, fat-soluble vitamins, water-soluble vitamins, calcium, magnesium, phosphorus, and aureomycin. Blood samples were obtained from jugular vein using a mixture of ammonium and potassium

TABLE I. Average Plasma Prothrombin Time Values of Matured Cows.

Breed	Herd No.	No. animals	Prothrombin time (sec.)
Jersey	1	8	20.63 $\pm$ 2.33*
	2	10	20.60 $\pm$ 3.43
	3	18	25.83 $\pm$ 2.54
	4	22	22.77 $\pm$ 3.78
	5	8	19.25 $\pm$ 1.25
	6	23	27.04 $\pm$ 5.28
	Total	89	Avg 23.74 $\pm$ 4.61
Ayrshire	5	9	20.67 $\pm$ 4.21
Guernsey	5	8	20.38 $\pm$ 2.27
Holstein	5	9	22.89 $\pm$ 2.03
All breeds	Total	115	Avg 23.20 $\pm$ 4.42

* Stand. dev.

oxalate (12) as the anticoagulant. In carrying out the one-stage plasma prothrombin time of Quick (2) 0.1 ml of plasma was pipetted into 7 by 100 mm pyrex test tubes. To the test tubes containing plasma, 0.1 ml of Bactothromboplastin[†] was added. Blood plasma and thromboplastin were mixed and placed in a thermostatically controlled water bath at 37°C. After 5 minutes, 0.1 ml of 0.02 M calcium chloride solution was blown forcibly from a pipette into the mixture and agitated by shaking gently in water bath. A stop watch was started simultaneously with addition of calcium chloride solution. At the end of approximately 10 seconds the tube was wiped free of water and held so that its contents were between the eye and a bright source of light. By rotating the test tube clockwise, it was possible to determine immediately when the solution began to clot and adhere to the wall of the test tube. Prothrombin time reported is that interval between addition of 0.02 M calcium chloride solution to the plasma-thromboplastin mixture and the first evidence of formation of a discernible clot or gel. Hematocrits were obtained by centrifuging 1 ml of oxalated blood from cows in Wintrobe hematocrit tubes for 30 minutes at 3000 rpm (13). Results were read directly from tubes as % packed erythrocytes.

Results. Plasma prothrombin time was obtained on 115 matured dairy cows and 90 dairy calves from 2 to 24 weeks of age. Table I shows that average prothrombin

[†] Difco Laboratories, Detroit, Mich.

time for 6 herds of Jerseys was 23.74 ± 4.61 . Analysis of variance showed that there was a highly significant difference (1% level) among herds. By calculating the significant mean difference among Jersey herds, the difference between herds 3 and 4 reached the 5% level of significance, differences between herds 1 and 3 and between 2 and 3 reached 1% level, and differences between herds 1 and 6, 2 and 6, 3 and 5, 4 and 6, and 5 and 6 reached 0.1% level of significance.

In analyzing data statistically of 4 breeds of cows in Table I, the F-value reached the 5% level of significance. The difference between the means for Jerseys and Guernseys was significant at the 5% level. The difference between Jerseys and Ayrshires approached the 5% level of significance.

According to Quick(2) plasma prothrombin time should be determined within 30 minutes after blood is collected, if accuracy is desired. A comparison was made of plasma prothrombin time values determined at 0.5, 2.5, and 3.5 hours after bleeding on 10 animals in herd 2. At these intervals prothrombin time values were 20.6 ± 3.43 ; 20.2 ± 3.80 ; and 21.0 ± 2.75 , respectively. These values were of no significance statistically when compared with 0.5 hour values.

Prothrombin time was obtained on plasma samples from 20 newborn calves prior to nursing. A clot or gel could not be detected in the test tube as usually observed. These test tubes were then placed in temperature controlled water bath for 2 hours, with periodic checking for clot formation. During this 2 hour period a clot did not form.

Table II shows plasma prothrombin time values of heifer calves 2 to 24 weeks old. There was no significant difference in prothrombin time values associated with age.

Plasma prothrombin time values of bulls and heifers were pooled to evaluate breed effects involving 90 bleedings. Average prothrombin time for Holstein calves was 25.15 ± 4.63 as compared with 24.55 ± 4.35 for Ayrshires. There is no significant difference between the values for Holstein and Ayrshire calves. Evidently rations did not alter prothrombin time values of calves significantly.

TABLE II. Average Plasma Prothrombin Time Values of Holstein and Ayrshire Calves at Various Ages.

Age (wk)	No. animals	Prothrombin time (sec.)	Range (sec.)
2	11	$26.91 \pm 4.51^*$	18-32
4	11	23.00 ± 5.53	16-30
6	12	25.92 ± 4.85	22-35
8	14	25.50 ± 3.63	20-33
12	13	24.85 ± 4.21	20-34
18	8	23.38 ± 4.47	20-30
24	4	26.50 ± 4.51	18-30
Total	73	Avg 25.11 ± 4.54	16-35

* Stand. dev.

Average prothrombin time for control calves was 24.80 ± 3.88 , for trace mineral supplemented calves 24.61 ± 4.23 , and for the vitamin-supplemented group 24.83 ± 5.27 . Trace minerals and other nutrients in addition to aureomycin did not delay or shorten prothrombin time of blood plasma.

Plasma prothrombin time values for Holstein and Ayrshire calves were pooled giving an average of 23.56 ± 3.96 seconds for bull calves and 25.10 ± 4.56 for heifers. These averages show a lower prothrombin time for bull calves than for heifers; however, in analyzing the data with the t-test no significant difference statistically was found between sexes.

In Table III the average hematocrit values for 92 matured cows are given, average $35.08 \pm 3.30\%$. There is an absence of breed effects but herd effects within the Jersey breed are significant at the 5% level. In calculating the significant differences among Jersey herds, a significant difference (5% level) was

TABLE III. Average Hematocrit Values of Matured Cows.

Breed	Herd No.	No. animals	Packed RBC (%)
Jersey	1	8	$32.50 \pm 1.41^*$
	2	10	35.05 ± 2.75
	3	18	35.56 ± 3.79
	4	22	35.45 ± 3.82
	5	8	36.13 ± 3.42
	Total	66	Avg 35.14 ± 3.48
Ayrshire	5	9	34.67 ± 2.50
Guernsey	5	8	36.00 ± 3.46
Holstein	5	9	34.22 ± 2.74
All breeds	Total	92	Avg 35.08 ± 3.30

* Stand. dev.

found between herds 1 and 3, 1 and 4, and 1 and 5.

Discussion. In Table I differences in plasma prothrombin time values among herds of Jersey breed and between Jersey and Guernsey breeds, that are of statistical importance, are probably influenced by rations. It has been shown in chickens(14) that feeding of terramycin lengthens blood clotting time. Other factors altering coagulation of blood are deficiency of vit. K(13), liver damage(13), poisonous plants(15,16), bacterial toxins(17), and halogenated hydrocarbons(18).

The plasma prothrombin time values for 10 animals fed trichloroethylene extracted soybeans(9) ranged from 21 to 28 sec., average 23.35. Averages reported in this paper are 23.20 on 115 matured cows and 24.82 on 90 calves. In no instance could the "incipient web of fibrin" be observed with blood plasma from cattle, as described by Quick (2) using human plasma, regardless of the number of variations placed upon the available light sources.

Numerous attempts were made to determine the plasma prothrombin time for calves immediately after parturition and before nursing. In all cases a clot did not form in the plasma with 0.02 M calcium chloride solution within 2 hours after mixing the contents of the test tubes. Sanford *et al.*(19) have shown that the % of prothrombin in blood plasma is slightly reduced in human infants. After birth the % of prothrombin is markedly reduced but the usual prothrombin time values are normally reached in 6 to 7 days. There is a similar trend in plasma prothrombin time of newborn calves. Greaves(20) and Bodansky(21) have attempted to explain the factors involved.

In some instances, it was found that a clot could not be obtained using a given plasma sample. Upon checking the time which had elapsed since the calcium chloride had been put into solution, it was found that 48 hours after preparation it was no longer effective in producing a clot in the mixture of plasma and thromboplastin. When a fresh solution of calcium chloride was prepared, a

prothrombin time value well within the range of other samples that had been similarly processed was obtained. According to Guerant(22) a change in the concentration of ionized calcium may have lengthened the prothrombin time. Wiggers(23) states that there is a maximum and minimum concentration of calcium chloride for the determination of plasma prothrombin time. When the concentration is not within this range, the prothrombin time is increased.

Summary. 1. The average plasma prothrombin time value of 115 matured dairy cows was 23.20 ± 4.42 and for 90 dairy calves 24.82 ± 4.47 . The differences in the prothrombin time values among 6 herds of Jersey cows were highly significant (1% level) and significant (5% level) among breeds between Jerseys and Guernseys. The principal factor contributing to these effects was ascribed to rations. Age, breed, ration, and sex did not alter the prothrombin time values of dairy calves. 2. The holding of the blood plasma samples for 2.5 and 3.5 hours after collection of blood samples did not change the prothrombin time significantly when compared with the 0.5 hour values. 3. The plasma prothrombin time of newborn calves, prior to nursing, could not be determined since a clot or gel did not form in the blood plasma-thromboplastin mixture after the addition of calcium chloride. This finding corresponds with the observations made with blood plasma from human infants during the first week of life. 4. The average hematocrit value of 92 dairy cows consisting of Jersey, Guernsey, Ayrshire, and Holstein breeds was 35.08 ± 3.30 . Breed differences in hematocrit values were not present; however, the differences among herds of the Jersey breed were significant at the 5% level.

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Hematogenous Pyelonephritis in Rats. II. Production of Chronic Pyelonephritis by *Escherichia coli*.* (22157)

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(Introduced by Carleton B. Chapman.)

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Development of chronic pyelonephritis in man is obscure because usually the disease is examined only in its end stages. The present experiments were undertaken therefore to provide an experimental model for a controlled study of processes which culminate in chronic pyelonephritis. A major objective was to observe effects of chronic infection on the kidney in the absence of hydronephrosis or other structural abnormalities. We described previously a simple, non-surgical technic for producing acute hematogenous pyelonephritis in rats, involving 2 steps; injection of *Escherichia coli* into blood, and massage of kidneys through intact body wall(1). The resulting disease was similar to acute pyelonephritis without manifest hydronephrosis in man, but

self-limited, and subsided in approximately 6 weeks. To produce chronic pyelonephritis, rats from this study were subjected repeatedly for several months to bacterial injection and renal massage. Special interest was directed to the following problems: 1) relationship of chronic pyelonephritis to vascular disease and hypertension, 2) progression of chronic renal inflammation after cessation of active infection.

Materials and methods. Sixty-one male Sprague-Dawley rats weighing 150 to 200 g each, were divided into 5 groups of 12.[†] All were fed rat chow[‡] and tap water *ad lib*. Treatments for each group are described in Table I. During the first 30 weeks, urine from rats in each group was obtained peri-

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[†] Group V consisted of 13 rats.

[‡] Uncle Johnny Mills, Houston, Texas.

TABLE I. Schedule of Inoculations and Renal Massage.

Group	Time of inoculation of <i>E. coli</i> (wk)	Total No. of <i>E. coli</i> inoculated	Time of massage (wk)
I	0	10 ⁸	Bilateral at 0, 9, 26
	7	10 ⁵	
	9	"	
	26	10 ⁸	
II	0	10 ⁸	Unilateral (right side) at 0 and 9; bilateral* at 26
	7	10 ⁵	
	9	"	
	26	10 ⁸	
III	None		Bilateral at 0, 9, 26
IV	0	10 ⁸	None
	7	10 ⁵	
	9	"	
	26	10 ⁸	
V	None		"

Inoculations made with 0.5 ml of 18 hr tryptose broth culture of *E. coli*. Strain used at 0, 7, and 9 wk was isolated from urine of patient with pyelonephritis and its virulence somewhat reduced by repeated subculture. The strain inoculated at 26 wk had been freshly isolated from blood of a second patient with pyelonephritis.

* Six of the 12 rats were given an additional inoculation of 10⁸ *E. coli* and bilateral massage at 12 wk.

odically by cystotomy for culture and microscopic examination. Blood was removed by cardiac puncture at these times for culture and measurement of urea nitrogen. Blood pressure of each rat was determined every 2 weeks by tail plethysmographic method described by Williams, Grollman, and Harrison (2), with the rat lightly anesthetized with ether. Surviving animals were sacrificed after 42 weeks and at autopsy, studies of urine and blood were repeated. In addition, half of each kidney was ground to a pulp with sterile sand and cultured on blood agar, EMB agar, and in tryptose broth. The remaining half, as well as portions of heart, liver, spleen, and bladder were fixed in 10% formalin. Sections were stained with hematoxylin-eosin and Schiff's periodic acid.

Results. Serial studies before sacrifice. Twelve rats from Groups I and II failed to survive to 42 weeks; causes of death were anesthesia accidents in 5, bacteremic shock in 3, and unknown in 4. *E. coli* was isolated from urine at least once during life in all but 3 of the 12 rats which survived to 42 weeks

TABLE II. Summary of Bacteriologic and Pathologic Findings at Time of Sacrifice (42 Weeks).

(Group)¶	No. surviving rats	Positive urine cultures	Positive kidney cultures (No. of kidneys = 2 × No. of rats)	BUN range and avg		Pyuria	
				During life	At sacrifice	Pyelonephritist	Pyuria No. with pyuria/No. of rats
I & II—r.m. and inoc. of <i>E. coli</i>	12	1 <i>E. coli</i> 3 <i>P. inter.</i> **	1 <i>E. coli</i> ¶ 6 <i>P. inter.</i>	17-42 (28.6)	24-38 (29.7)		6/12§
III—r.m. only	10	0	2 <i>P. inter.</i> *	25-33 (28.3)	26-34 (30.3)		0/10
IV—inoc. of <i>E. coli</i> without r.m.	6	0	0	20-33 (28.5)	Not done		1/6
V—controls; no inoc. or r.m.	13	0	4 <i>P. inter.</i> *	17-40 (28.9)	24-27 (25.5)		0/13

* Isolation of *P. intermedium* probably resulted from blood in the kidney and not from renal tissues themselves since urines were negative and pyelonephritis was not present in these animals.
† Numerator = No. of kidneys with pyelonephritis; denominator = total No. of kidneys examined.
‡ Questionable pyelonephritis only (see text).
§ Includes the 4 rats with positive cultures.
¶ r.m. = renal massage.
** *P. inter.* = *P. intermedium*.
¶ One of the kidneys in this animal was sterile.



FIG. 1. Hilar cross sections of both kidneys from the same animal in Group 1 ($\times 4$). Note marked differences in size, gross distortion produced by scarring, and loss of cortical substance. Urine culture had been positive for *E. coli* at 5 wk but both urine and kidney cultures were negative at 42 wk.

and in 6 dying earlier. Six rats in Group IV survived 42 weeks after the first inoculation of *E. coli*. Urines from 3 animals yielded positive cultures after first inoculation, but all subsequent urines from these and remainder of the group were negative. Two rats

died of bacteremic shock and 2 died of unknown causes. Two were sacrificed at 36 weeks and negative cultures obtained from both urine and kidneys. In Group III, 2 deaths occurred from unknown causes; in Group V, all survived. All urine cultures in

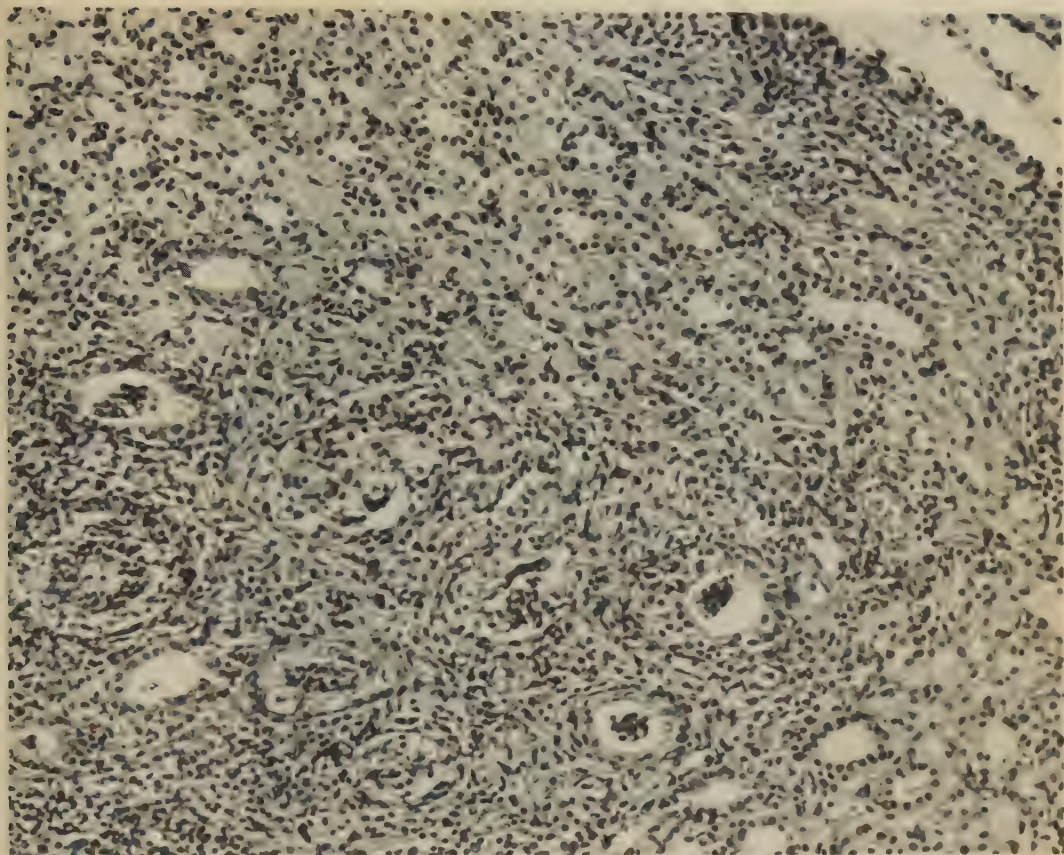


FIG. 2. Microscopic section of papillary tip of pyelonephritic kidney. Note fibrosis, mononuclear infiltration, and dilated tubules containing pus casts ($\times 140$). Urine cultures were positive for *E. coli* at 10 and 30 wk; *P. intermedium* was cultured from urine and kidneys at 42 wk.

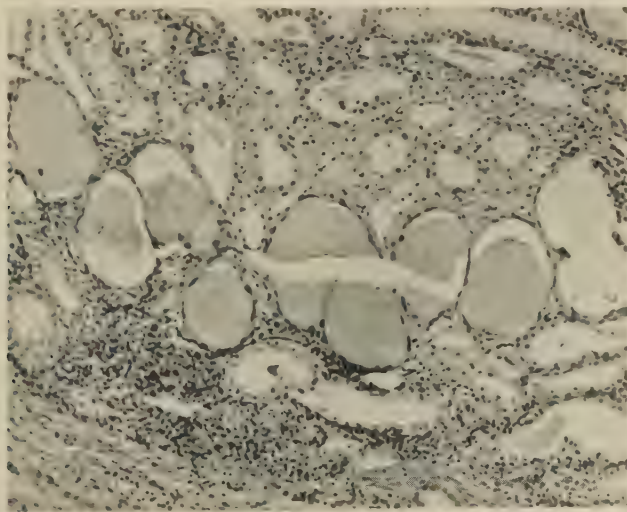


FIG. 3. Microscopic section from medullary area of pyelonephritic kidney revealing dilated, colloid-filled tubules. At the lower left, an area of fibrosis and mononuclear infiltration extends through medulla ($\times 65$). Urine culture positive for *E. coli* at 5 wk and negative at 15 and 42 wk; kidney culture negative at 42 wk.

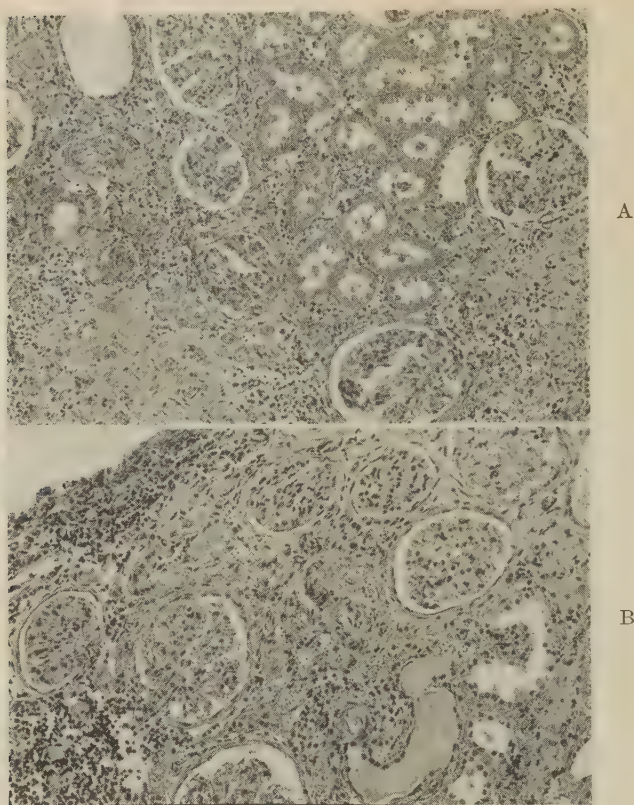


FIG. 4. Microscopic sections from 2 areas of cortex of pyelonephritic kidneys. A. Intact glomeruli with periglomerular fibrosis and marked interstitial fibrosis. Note interstitial accumulation of lymphocytes and plasma cells, and normal appearance of arterioles ($\times 65$). Cultures of urine and kidneys negative at 42 wk. B. Marked interstitial infiltration with mononuclear cells and widespread periglomerular fibrosis. Glomeruli themselves are normal ($\times 70$). Urine culture positive for *E. coli* at 5 wk, negative at 15 and 42 wk. Culture of kidney negative at 42 wk.

both groups were negative. Blood cultures repeatedly were negative in all five groups. Blood urea nitrogen was normal; values are listed in Table II.

Studies at completion of experiment. Bacteriological and pathological findings in all animals when sacrificed (42 weeks) are summarized in Table II. The 12 surviving animals in Groups I and II have been considered as a single group because all were subjected ultimately to bilateral renal massage. *E. coli* could still be isolated from only one rat in this combined group. From pyelonephritic kidneys and urine of 3 additional animals in the group, *Paracolobactrum intermedium* was cultured.

Pathologic findings. 1) Kidneys (Fig. 1-4). Changes of chronic pyelonephritis were found

in all 12 rats in Groups I and II which survived 42 weeks (Table II). Four of the 12 animals dying earlier were examined, and 2 displayed pyelonephritic changes. The 2 in whom changes were absent had died after 12th week of the experiment. Kidneys were often adherent to surrounding organs and usually differed in size. They were distorted, lobulated by scars, and displayed loss of cortical substance and increased thickness of pelvic walls. Microscopic examination revealed areas of interstitial inflammation consisting of lymphocytes and plasma cells which extended throughout cortex and medulla. The tubules were dilated and many contained large colloid casts ("thyroid kidney"). Numerous "pus casts" were observed in kidneys from which positive cultures were obtained,

but only a few in absence of demonstrable infection. There was extensive interstitial and periglomerular fibrosis but glomeruli themselves usually appeared normal. Significant vascular lesions were absent. Thickness of media of arterioles never exceeded that in controls. There was no intimal proliferation or medial necrosis. Rarely, an arteriole in the midst of a dense scar showed slight hyalinization. In 2 rats from Group IV, minimal findings of pyelonephritis were present, consisting of scattered infiltrations with lymphocytes and plasma cells and slight fibrosis; a single, small scar was observed grossly in one kidney. Among the remaining control animals (Groups III and V), occasional tiny depressions on surface of kidney were seen grossly, which corresponded to small areas of cellular infiltration beneath the capsule. A few tubules were dilated and contained colloid, but never to the degree seen in Groups I and II. In 2 rats with sterile kidneys, rare areas of cellular infiltration without fibrosis suggesting pyelonephritis were observed in cortex and medulla. 2) *Other organs.* Tiny well-healed scars were present in the hearts of many animals at site of needle puncture, but there was no other abnormality. Bladders in Groups I and II were often slightly thickened. Significant changes in spleen and liver were absent, except for splenic enlargement in animals with septicemia. *Blood pressure.* Mean systolic blood pressures and standard deviations of each group were: Group I and II: 111 ± 6 ; Group III: 116 ± 4 ; Group IV: 109 ± 5 ; Group V: 113 ± 6 mm Hg, respectively. Average blood pressures of pyelonephritic rats were thus not significantly higher than controls and hypertension was not observed in any individual animal, including those which died prior to completion of the study. Single determinations as high as 140 mm Hg[§] were noted

rarely in animals from all groups but these were not sustained.

Discussion. Pathologic changes in kidneys of rats in Groups I and II closely resembled those seen in chronic pyelonephritis in man (3). The technic therefore provides an experimental model for examining problems related to the human disease.

The data suggest that recurrent, self-limited renal infections lead to permanent renal damage and inflammation which may persist even after infection has terminated. Although polymorphonuclear infiltration of kidneys and pyuria tended to diminish upon disappearance of active infection, an inflammatory reaction remained, characterized by infiltration of plasma cells and lymphocytes and accompanied by proliferation of connective tissue. Studies are presently underway to determine whether these sterile inflammatory lesions gradually subside or progress in the absence of reinfection and lead to renal failure.

Bacteremia due to *Paracolobactrum intermedium* noted in 3 control animals failed to produce pyelonephritis or infected urine. In 3 pyelonephritic rats in Groups I and II, however, *P. intermedium* was associated with pyuria and infected urine. These data suggest that the diseased kidney is rendered more vulnerable to infection by secondary invaders.

In contrast to the impression gained by investigators from clinical-pathologic correlations in the human (3), there was no evidence in these animals that hypertensive vascular disease develops as a direct sequel to the inflammatory process. Although extensive pathological damage was produced in these rats, areas of normal kidney were present and sufficient renal function remained to prevent azotemia. Observations over longer periods should help to determine whether more severe impairment of renal function must develop or some other factor affecting arterioles must be imposed before vascular disease appears.

Summary and conclusions. 1) Chronic bilateral pyelonephritis without manifest hydronephrosis was produced experimentally in the albino rat by a simple non-surgical technique. This experimental disease provides a model for study of human pyelonephritis. 2)

§ The average systolic blood pressure of normal Sprague-Dawley rats as determined with the tail plethysmographic method in this laboratory is 110 mm Hg with a standard deviation of ± 10 . A rise of 5 to 10 mm Hg occurs with age. Rats in whom experimental hypertension is produced by renal ischemia reveal blood pressures of 150 mm Hg and above, with this method.

Renal inflammation tended to persist after infection had disappeared. 3) Vascular disease and hypertension did not develop; however, the lesions were of insufficient severity to produce azotemia.

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Purification of Hyaluronidase from *Clostridium perfringens*.^{*} (22158)

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Although several investigators(1-4) have prepared highly purified hyaluronidase from bovine testicular tissue, bacterial hyaluronidases have not usually been purified to a comparable degree. Most authors(5) have used either simple filtrates of the bacteria or crude $(\text{NH}_4)_2\text{SO}_4$ precipitates. The notable exception was the discovery by Rogers(6) that crude precipitates from ammonium sulphate treatment of filtrates of *Clostridium perfringens*, Type A, Strain 107, could be absorbed on aluminum oxide at pH 4.6 and eluted at pH 7.0 to give a 16-fold increase in potency of hyaluronidase preparations. This method is difficult to reproduce and Rogers was not always able to get a sharp separation with his preparations. For this reason we initiated an alternate method for purification of the bacterial hyaluronidases. This report deals with the preparation of highly purified hyaluronidase from *Cl. perfringens*, culture 2278.

Methods. The hyaluronidase unit was based on the unit of testicular hyaluronidase previously reported(7). Due to instability of the bacterial filtrates at pH 5.5, the enzyme

was incubated with hyaluronic acid at pH 7.0. Fig. 1 shows that turbidimetric curves coincided in the range of 2 to 5 units. Fractions for assay were diluted with a buffered solution of 0.1% bovine serum albumin(8) until they fell within these limits. Nitrogen content of various fractions was determined by micro-Kjeldahl digestion with selenium-sulfuric acid followed by nesslerization. Three strains of *Clostridium perfringens* were studied. A lyophilized stock strain, #47-E-2, Walter Reed Army Institute of Research collection, represented the so-called laboratory strains. It had been held in dried state since 1944, but there were no records of its source. A second culture, #4209, isolated in 1951,

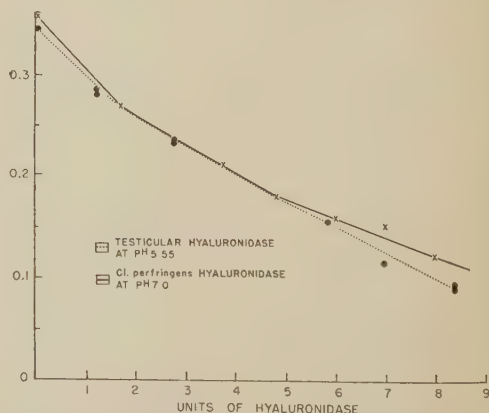


FIG. 1. Comparison of relationship of optical density to *Cl. perfringens* hyaluronidase and testicular hyaluronidase.

^{*} Presented in part before the Soc. of Am. Bact., Pittsburgh, Pa., May 1954.

[†] Abstracted in part from thesis presented to Graduate School, Georgetown University, in partial fulfillment of requirements for the degree of Doctor of Philosophy.

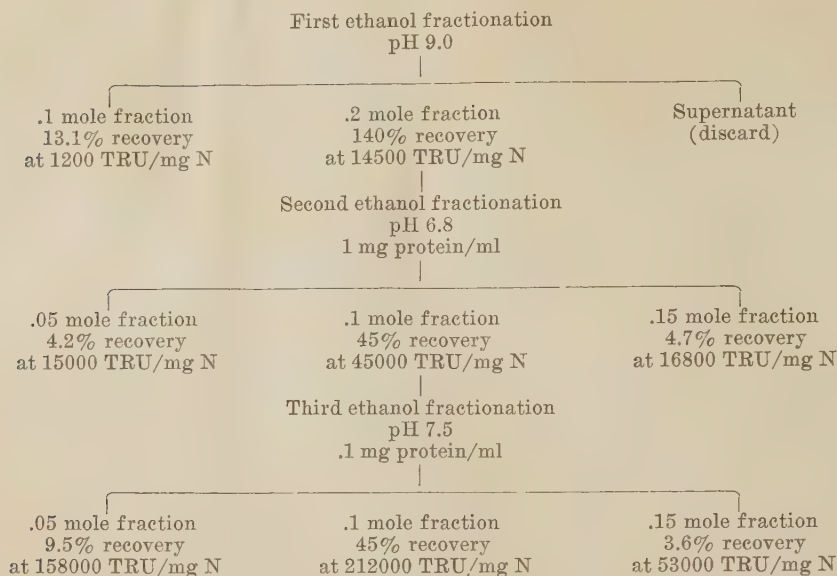
from a gangrenous war wound represented a strain of known pathogenicity. It was isolated and lyophilized at the 406th Medical General Laboratory (Tokyo) approximately one year prior to these studies. A third strain, #2278, had been isolated from the gangrenous wound of a patient at Walter Reed Army Hospital in 1952. This culture had been maintained on blood agar plates in an anaerobic atmosphere and transferred at monthly intervals.

Results. Choice of strain and medium. Considering the nutritional selectivity of *Clostridia* 3 types of culture media, at pH 7.6, were studied to obtain the greatest amount of bacterial filtrate: Difco brain heart infusion broth containing 1.5% yeast extract, Bernheimer's synthetic medium, and trypticase lactose iron broth. Starter cultures for mass inoculation were prepared by seeding 1 liter of each of these media with 10 ml of thioglycollate broth from the blood agar plates on which the various strains had been initially recovered from stock. Good growth was obtained after 16 hours incubation at 35°C. One liter quantities of the 3 selective media were prepared in the usual manner. Excess oxygen was displaced by boiling and cooling to approximately 40°C immediately prior to adding 10 ml of thioglycollate broth starter culture. The 1 liter flasks were next sealed with parafilm and incubated at 35°C for 18 hours, after which the cultures were centrifuged. Supernatants were adjusted to pH 9.0 with 1N sodium hydroxide and sterilized by Seitz filtration.

Hyaluronidase yields by strains of *Clostridia* employed were widely divergent. All strains failed to grow in Bernheimer's synthetic medium. Fair growth was obtained in trypticase lactose iron broth, but hyaluronidase yield was not satisfactory. The failure of both media to support good growth of *Clostridium perfringens* was unexpected. Best results were obtained in brain heart infusion broth with 1.5% yeast extract added. The hyaluronidase yield in this medium was 10 times that obtained in trypticase lactose iron broth and gave values of 0, 60 and 100

units/ml of culture filtrate for strains #47-E-2, #4209 and #2278 respectively. Within the small number of strains tested, there appeared to be a correlation between length of time these cultures have been held in the dried state and their ability to produce hyaluronidase. Culture #47-E-2, in the dried state for more than 10 years, failed to produce the enzyme, although it grew well. Culture #4209, lyophilized in 1951, produced small amounts of hyaluronidase. On the other hand, culture #2278, which had never been lyophilized, produced by far the most enzyme. This culture was used for subsequent preparations of purified hyaluronidase.

Preparation of purified hyaluronidase. Preliminary attempts to fractionate the bacterial filtrate at pH 7.6 were completely unsuccessful. The enzyme appeared to be stable in the filtrate at this pH for only 72 hours and then was rapidly destroyed. Precipitation with 0.7 ammonium sulphate or with ethanol appeared to hasten destruction of the enzyme. Stabilization of the original culture filtrates, and the first crude fractions, was necessary. Since instability of the hyaluronidase might be due to proteolysis by other enzymes in the culture filtrates, the effect of pH on stability of the hyaluronidase at 0°C was investigated, using the culture filtrates and the 0.7 saturated ammonium sulphate precipitate buffered to several pH values with and without ethanol at 0.3 mole fractions. The 0.3 mole fraction of ethanol was selected to represent maximum concentration of ethanol anticipated in subsequent experiments. Below pH 7.0 destruction of the enzymes was exceedingly rapid. At pH 7.0 in the buffer, the enzyme showed a minor loss in 1 hour but was completely destroyed in 24 hours. In ethanol it was completely lost. Between pH 8.6 and 9.5 the enzyme remained indefinitely stable in either buffer or buffer containing ethanol. At lower hydrogen ion concentrations only the buffered enzyme remained stable. Consequently, the cultures of *Cl. perfringens* were immediately adjusted to pH 9.0 after the bulk of organisms had been removed by centrifugation at 4000 rpm and supernatant clarified by

FIG. 2. Ethanol fractionation of hyaluronidase from *Cl. perfringens*.

Seitz filtration. Solid ammonium sulphate was added to the clear filtrate to 0.3 saturation. The suspension was allowed to stand 24 hours at 4°C. The inactive precipitate was removed by centrifugation and discarded. Ammonium sulphate was added to the supernate to 0.7 saturation, and the suspension was again allowed to equilibrate for 24 hours at 4°C. The active precipitate was removed by centrifugation and dissolved in one-fifth the original volume with 0.02 molar veronal buffer, pH 9.0, containing 0.45% NaCl.

The ethanol fractionations were conducted as previously described(2) except that suspensions were allowed to equilibrate for 24 hours after addition of each increment of ethanol and precipitates removed in refrigerated centrifuge, -5°C, at 4000 rpm. Application of this technic to testicular hyaluronidase yielded 15 to 25% recovery of the crude enzyme and a purified preparation assaying 160000 TRU/mg N. Typical results of the first ethanol fractionation are shown in Fig. 2. The recovery figures are based on the hyaluronidase content of 3.5 l of culture filtrate which contained 100 TRU/ml at an index of purification of 200 TRU/mg N. The ammonium sulphate precipitation in combination with the first ethanol fractionation there-

fore caused a 70-fold purification of the bacterial hyaluronidase. Recovery in the dialyzed 0.1 and 0.2 mole fractions of 153% of the total enzyme, can be partly explained by the increased stability of these fractions at pH 7.0 where the assay was conducted. The apparent anomaly may also be due to removal of inhibitors present in the original culture filtrate. As this stage the 0.2 mole fraction was stable indefinitely at pH 7.3 in buffered solution and was stable for at least 72 hours at pH 7.0.

Previous work suggested that the greatest purification could be achieved by fractionating next at a low pH. Consequently, pH 6.8 was chosen for the second ethanol precipitation. The 0.2 mole fraction from the first ethanol was dialyzed, and concentrated phosphate buffer containing NaCl was added to 0.02 M phosphate, pH 7.3 containing 0.45% NaCl. The solution was diluted with buffer to 1 mg protein/ml, and the pH adjusted to 6.8. Fig. 2 shows destruction of the enzyme at this pH since only 54% was recovered. However, the 0.1 mole fraction contained 45% of the original enzyme 3 times purer.

The third ethanol fractionation was conducted at pH 7.5. The 0.1 mole fraction from the second ethanol treatment was dis-

solved in the 0.02 M phosphate buffer pH 7.3, diluted to 0.1 mg protein/ml and pH was adjusted to 7.5. It is believed that the 4-5 fold increase in purification shown in Fig. 2, is due more to the very dilute solution of protein fractionated rather than change in pH. As in the first ethanol fractionation over 100% recovery of the enzyme being fractionated was obtained. This increase could not be attributed to increased stability of the enzyme at assay pH of 7.0, because the enzyme was stable at this pH for at least 3 weeks after the second ethanol fractionation. It seems more likely to be due to removal of inhibitors during the purification procedure.

An apparent over-all recovery of 45% was obtained by this procedure and the enzyme was purified 1000-fold. However, it is obvious from the data that removal of inhibitors during purification may account for part of this high yield. In small scale experiments, it appeared that if the second ethanol was conducted at pH 7.0 instead of 6.8 even higher yields could be obtained.

Rogers(6,9) does not mention the loss of *Cl. perfringens* hyaluronidase below pH 7.0. In fact, we may assume that this did not occur with his strain since it was absorbed on aluminum oxide at pH 4.6 (a pH which would have caused immediate destruction of hyaluronidase preparations obtained in these laboratories). The marked difference in sta-

bility of these two hyaluronidase preparations cannot be explained at present. Conditions of growth, as well as strains, permit many possibilities. However, it is suggested that two hyaluronidase disaccharases may be present in these organisms. Consequently, the two laboratories may not have been dealing with the same enzyme.

Summary. A procedure has been detailed for a 1000-fold purification of a bacterial hyaluronidase from *Cl. perfringens*, strain 2278. Initial crude fractionation with ammonium sulphate followed by three ethanol fractionations at pH 9.0, 6.8 and 7.5 respectively have yielded hyaluronidase fractions containing 200000 TRU/mg N with recovery of 45% of the crude enzyme.

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Production of Impending Hepatic Coma by a Carbonic Anhydrase Inhibitor, Diamox.*† (22159)

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Impending hepatic coma, characterized by confusion and the typical "flapping" tremor, has been described previously in alcoholics with cirrhosis given oral nitrogenous sub-

stances—ammonium cation exchange resins, ammonium salts, urea, and increments in dietary protein(1-3). The onset of this syndrome is here described in similar patients following oral administration of a carbonic anhydrase

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† A preliminary report is included in the *Proc. Am. Soc. Clin. Investig.*, May 1955.

TABLE I. Clinical and Laboratory Data of 12 Alcoholics with Cirrhosis Given an Oral Carbonic Anhydrase Inhibitor, Diamox.

Patient, age, sex	BSP†/Ser. bil. (mg/100 ml)	Diamox-Dose, frequency,‡ duration	KCl daily dose (g)	Mental state during Diamox‡	“Flapping” tremor dur- ing Diamox	Avg periph. vein blood NH ₃ -N** (γ/100 ml)	
						Before§	During
Group I (Developed impending coma during Diamox)							
J.H.,* 49, ♀	35/ 1.2	mg Days					
		500 A 3	0	C	Appeared	—	—
		" A 13	6	C	"	51 (2)	138 (8)
		" D 6	6	C	"	81 (2)	105 (4)
Started 3rd day							
J.M.,*¶ 32, ♂	36/ 4.5	" A 3	0	C	"	—	—
J.S.,¶ 44, ♂	—/ 8.0	" D 7	3	C	More severe	65 (3)	116 (4)
J.T.,¶ 50, ♂	33/ 3.5	1000 D 5	0	C	0	55 (3)	50 (3)
Group II (No impending coma)							
H.D.,* 40, ♀	48/10	500 A 27	0	Dr	0	63 (4)	87 (17)
	16/ 1.2	" D 3	0	N	0	78 (1)	69 (1)
E.F.,* 37, ♀	—/ 4.3	" A 27	0	N	0	62 (3)	87 (19)
J.Mc.,* 45, ♂	25/ 1.3	" D 7	0	N	0	74 (2)	89 (5)
G.A., 52, ♂	—/15.6	" D 6	0	N	0	85 (4)	92 (4)
F.J., 62, ♂	31/ 2.0	" D 8	0	N	0	59 (3)	66 (4)
C.O'D., 49, ♀	29/	500 D 7	0	Dr	0	73 (4)	88 (6)
		Then 1000 D 2					
G.D., 42, ♀	51/ 4.7	1000 D 7	0	N	0	64 (3)	73 (4)
M.B., 47, ♀	—/ 6.9	" D 5	0	Dr	0	48 (2)	55 (4)

* Diagnosis of Laennec's cirrhosis confirmed by postmortem or liver biopsy.

† Bromsulfalein: % retention in 45 min.

‡ A = Alternate days; D = Daily; C = Confused; Dr = Drowsy; N = Normal.

§ Before Diamox administration.

|| During " " "

¶ History of previous mental symptoms associated with liver disease.

** Numbers in parentheses represent the number of determinations.

inhibitor, acetazolamide (Diamox)†(4,5). This drug, as well as sulfanilamide, has been employed in the treatment of cardiac failure because it may cause a sodium and water diuresis(6,7).

Materials and methods. From January, 1954, through January, 1955, on the Thorndike Ward, 12 alcoholic patients with cirrhosis received 15 courses of Diamox therapy (Table I). One patient received 3 courses, another 2. Six patients were men and 6 women, ranging from 32 to 62 years of age. Diagnosis of cirrhosis was made by history of prolonged alcohol and poor food intake coupled with physical and laboratory evidence consistent with severe long-standing liver disease. Six of the 12 patients had mild hemolytic anemias thought to be consistent with liver disease(8) and 3 had peripheral

neuritis. Rheumatic heart disease, treated "beri-beri" heart disease, and psoriasis were diagnosed in one instance each. Diagnosis of cirrhosis was confirmed by postmortem examination in patients J. M. and J. H., who died several months later, and by liver biopsy in 3 other patients. One of the latter, J. Mc., also had peritoneal tuberculosis when biopsy was performed 3 months after Diamox. All patients had fluid accumulation and received weighed diets containing less than 270 mg of sodium and from 66 to 75 g of protein daily. An oral vitamin supplement Vi Magna Granules,§ containing vit. A, B complex, C, and D, was added daily. Stools were examined frequently for occult blood. Blood was drawn in the fasting state or 2 to 3 hours postprandially in the morning. Hematocrits, serum carbon dioxide contents, and concentrations

‡ Provided through the courtesy of Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

§ Provided through the courtesy of J. M. Rueggsegger of Lederle Laboratories Division of American Cyanamid Co., Pearl River, N. Y.

of nonprotein nitrogen, sodium, potassium, and chloride, were determined using methods previously described(9). Serum bilirubin, 45-minute bromsulphalein retention, cephalin flocculation, thymol turbidity and flocculation were also determined using standard methods. One ml of peripheral venous blood drawn in a heparinized syringe was immediately injected into the alkaline outer chambers of each of 2 Conway units and analyzed for "ammonia" nitrogen ($\text{NH}_3\text{-N}$)^{||} using McDermott and Adams' modification of the Conway technic(10). Our values were lower than those of McDermott *et al.*, but recoveries from standard solutions were good. The normal range was from 29 to 86 mg $\text{NH}_3\text{-N}$ per 100 ml (16 patients, 19 determinations). Before Diamox 71 control determinations in 12 patients with cirrhosis ranged from 28 to 114 μg per 100 ml with only 2 determinations exceeding 100. Eight patients with spontaneous impending hepatic coma, not included here, had concentrations (21 determinations) ranging from 35 to 246 μg per 100 ml and 6 of these were below 114. Blood $\text{NH}_3\text{-N}$ determinations during Diamox administration were made on the morning following initial dose and most mornings thereafter up to and including the morning the drug was discontinued. Concentrations presented (Table) represent averages; figures in parentheses represent the number of determinations from which averages were calculated. After blood $\text{NH}_3\text{-N}$ determinations were made, Diamox was given in amounts of from 500 mg on alternate days to 1000 mg daily from 3 to 27 days. Potassium chloride was given in daily oral quantities of 3 g to one patient (J.S.) and 6 g to another (J.H.); the latter received it before and during one course of Diamox and also during part of another (Table).

Confusion and the typical "flapping" tremor(11) were not present before Diamox except in J. S., who had a slight tremor.

Results. Two groups were established

^{||} Ammonium ion is either present in shed blood or formed from nitrogenous substances in blood shortly after shedding. Volatile blood base, liberated by adding strong alkali, is probably all or nearly all ammonia.

(Table): Group I, 4 patients who developed confusion with or without tremor, and Group II, the remaining 8, none of whom developed this syndrome. All in Group I had chronic severe cirrhosis and 3 had previously had impending hepatic coma. Of the 8 in Group II, 5 had cirrhosis of a severity comparable to those in Group I, but none had had impending coma.

Beginning within 2 to 48 hours of the initial dose, 4 patients developed confusion, during 6 periods of Diamox administration, 2 after only 500 mg. Tremor remained absent during one period, became accentuated in one, and appeared in 4. Confusion became marked in 2 patients (J.H. and J.M.) and remained mild in the other 2. Except in J.M. it did not progress after the first 48 hours and improved in J.S. and J.T. despite continuation of Diamox. The tremor usually persisted longer.

During Diamox administration average venous blood $\text{NH}_3\text{-N}$ concentrations rose above those of the highest control determination (114 μg per 100 ml) during 2 of 4 periods. During Diamox, average $\text{NH}_3\text{-N}$ concentrations and increases in these 2 instances exceeded all control averages in Group II at the 5% level or less. Concentrations fluctuated from day to day and were usually higher in the first 72 hours, falling later despite continuation of Diamox. Average serum potassium concentrations decreased during all 6 periods, during 4, 0.4 mEq or more. Potassium chloride, administered during 3 periods, failed to prevent or alleviate impending coma although average serum potassium concentrations remained above 4 mEq per l in patients who received it.

In Group II, confusion and tremor were absent, although drowsiness was present in 3 of 8 patients during Diamox administration. Average blood $\text{NH}_3\text{-N}$ concentrations increased during all except one period. Day to day values, highest in the first 72 hours, fluctuated, sometimes reaching abnormally high levels. In one of 7 periods the average serum potassium concentration decreased more than 0.4 mEq per l, and during another a low value (2.7 mEq per ml) was found.

The 2 groups showed no significant difference in serum carbon dioxide contents or concentrations of sodium and chloride before or after Diamox. Serum sodium concentrations rose slightly during 6 and remained constant during 8 periods of Diamox therapy; serum chloride concentrations rose during 9 periods, remained the same during 3, and dropped slightly during one. Serum carbon dioxide contents decreased during 11 periods and remained the same in 3. Acidosis (a rise in serum chloride concentration and fall in carbon dioxide content) occurred even when the smallest doses of Diamox were given.

Discussion. Clinical signs and symptoms which may occur after Diamox administration are well documented by Maren *et al.*(12). Noted in patients without liver disease have been paresthesias, drowsiness, and deep sleep. Confusion was not reported and we did not observe it in 12 patients given single 500 mg oral doses of Diamox.

In cirrhosis of the liver, however, bizarre changes of mood, hallucinations, loss of memory, confusion, and occasional psychotic episodes were first described by Eisenmenger (13) to occur following Diamox administration. Confusion also appeared here, but was, in addition, often associated with the typical "flapping" tremor which occurs in impending hepatic coma.

Impending hepatic coma, characterized by confusion and "flapping" tremor in patients with liver disease, is often but not always associated with increased venous blood $\text{NH}_3\text{-N}$ concentrations(14). Two of our 3 patients with confusion and tremor had significant increases and elevated concentrations of blood $\text{NH}_3\text{-N}$ after Diamox. The nitrogen contained in Diamox was not the source of increased blood $\text{NH}_3\text{-N}$ because in cirrhosis this drug is nearly all excreted in the urine(13). The pathogenesis of impending hepatic coma after Diamox is different, then, from that induced by nitrogenous substances thought to liberate ammonium in the gastrointestinal tract.

Diamox may have a direct effect on brain metabolism because parasthesias occurring in the first 8 hours after Diamox improved as

the plasma concentrations of Diamox could be predicted to be falling and because administration of sulfanilamide, another carbonic anhydrase inhibitor, produced confusion in a large percentage of patients without liver disease(12). The mechanism of action is unknown, although sulfanilamide interferes with maintenance of potassium gradients in guinea pig brain slices(15). Decreases in serum potassium concentrations were more marked here in the group developing impending coma, but mental symptoms occurred at normal serum potassium concentrations and potassium salts produced no improvement. Possibly, the brain of patients with severe hepatic disease is more sensitive to Diamox because only these patients developed confusion in our hands.

Conclusions. 1. The carbonic anhydrase inhibitor, Diamox, can produce impending hepatic coma in susceptible alcoholics with cirrhosis. 2. This type of induced impending coma may be associated with a rise in femoral or antecubital vein blood ammonia nitrogen concentrations to levels above those seen in mentally clear patients with cirrhosis. 3. The nitrogen contained in Diamox is not a source of the elevated blood ammonia nitrogen concentrations.

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Residual Organ Blood Volume of Cattle, Sheep and Swine.* (22160)

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In quantitative studies involving investigations of tissue distribution of blood-borne substances a correction for the residual organ blood may be essential. This is of special importance in short time radioisotope distribution experiments where retained tissue blood may be sufficient to contribute significantly to the total activity recovered. Specific studies based upon whole tissue extractions measure not only the substance in the cells of the tissues, but also that existing in the blood which is trapped within these tissues at the time of sampling. Therefore, in tracing the distribution of an ingested or injected substance by extraction from various organs, it is necessary to know the proportion of the material that is from the tissue and that which is from the blood itself. When the relative proportions of blood and tissue are known, precise quantitative studies on movement and tissue composition may be used to substantiate microscopic examinations and organ weight changes. The total organ weights for farm animals have been reported by several investigators(1-3), and procedures and values for blood volume of cattle, sheep and swine are in the literature(4-6). Lewis *et al.*(7) have reviewed procedures and reported the total or-

gan blood volume measurements for the rat. Other investigators have made *in vitro* and *in vivo* measurements of total organ blood volumes in laboratory animals(8,9) but there were no data available from the literature directly related to the residual blood present in organs and tissues of farm animals slaughtered by the conventional procedures.

This report is concerned with the residual blood values as related to age and species for the various organs and tissues of farm animals routinely sacrificed and bled out during current nutritional investigations.

Material and methods. The cattle, sheep and swine used in these studies were normal, healthy individual animals maintained under general farm management conditions. Total red blood cell volumes were obtained by the injection of P³² labeled homologous erythrocytes as previously described(4). The organ blood values were procured as follows: (a) blood samples were drawn from the animal; (b) whole blood was incubated with P³²; (c) red blood cells were washed sufficiently to remove any radioactivity not in the cells and (d) the cells were reconstituted to the original volume with the animal's own plasma. (e) A known volume of suspended labeled cells was re-injected into the immobilized animal(10) and after mixing had occurred, blood samples were taken from the jugular vein of the animal, usually at 5 and at 15 minute intervals for radioactivity measurement and blood volume determinations. (f) The animal was then sacrificed by a blow

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TABLE I. Mean Organ Weights for Cattle, Swine and Sheep.

Species	Age (mo)	No. of animals	Mean wt (lb)	Fresh wt as % of body wt $\pm$ stand. dev.					
				Blood*	Liver	Kidney†	Spleen	Lungs	Heart
Cattle	.5-3	12	88	8.4	2.31 $\pm$.71	.230 $\pm$.035	.359 $\pm$.106	1.57 $\pm$.42	.767 $\pm$.242
	6	15	384	6.0	2.24 $\pm$.19	.124 $\pm$.024	.266 $\pm$.021	.821 $\pm$.12	.457 $\pm$.087
	Aged	8	923	5.6	2.17 $\pm$.06	.130 $\pm$.032	.256 $\pm$.024	.859 $\pm$.120	.471 $\pm$.051
Sheep	.5	5	10	9.3	1.69 $\pm$.24	.560 $\pm$.30	.320 $\pm$.28	1.65 $\pm$.05	.740 $\pm$.01
	4	6	80	6.5	1.84 $\pm$.24	.140 $\pm$.02	.240 $\pm$.03	1.08 $\pm$.10	.360 $\pm$.12
	Yearlings	8	118	5.9	1.30 $\pm$.29	.120 $\pm$.02	.260 $\pm$.02	1.00 $\pm$.30	.390 $\pm$.08
Swine	.5	7	10	7.2	2.52 $\pm$.25	.415 $\pm$.046	.297 $\pm$.125	1.29 $\pm$.22	.609 $\pm$.163
	2	11	35	6.7	2.10 $\pm$.26	.299 $\pm$.048	.220 $\pm$.066	1.21 $\pm$.22	.472 $\pm$.091
	5	6	102	6.3	2.66 $\pm$.34	.242 $\pm$.061	.232 $\pm$.051	.95 $\pm$.20	.403 $\pm$.101
	Aged	6	654	3.5	1.859 $\pm$.22	.820 $\pm$.0228	.218 $\pm$.029	.834 $\pm$.117	.319 $\pm$.039

* ml of blood/100 g body wt.

† Mean wt of one kidney only.

on the head, hoisted by the rear legs and bled out for 5 minutes. The organs and tissues were immediately excised, blotted free of excess blood and weighed, (g) whole organs or 10 to 15 samples were taken from each organ or tissue being studied for the measurement of radioactivity, and (h) these average values were used for the estimation of the residual blood present. All tissue samples were weighed and wet ashed with nitric acid, made to volume and aliquots taken for radioactivity measurements by conventional procedures as described by Hansard *et al.* (4). The milliliters of blood per 100 g of tissue were then calculated from the isotope concentration in the samples and the blood volume standard (5).

Results. Organ weights of farm animals. The mean unit weights of the various organs of cattle, sheep and swine employed are listed in Table I as a percentage of the live body

weight. These average values compare favorably with those reported by other investigators (1-3). It is noted that there was more difference due to weight of animal than to species. The very young of all species showed a higher blood volume and slightly higher mean organ weights. These values are included to provide a means of estimating the total residual blood present in whole organs and tissues.

Residual blood in cattle organs and tissue. The residual blood in organs of 7 cattle in 2 age groups is shown in Table II. There was a wide variation between individual tissues but the total blood volume values were similar to those reported previously (4). In these animals the spleen retained the highest concentration of residual blood, followed by the lungs, liver, kidney, pituitary, adrenal, heart, pancreas and muscle in that order. Age appeared to affect concentration of retained

TABLE II. Residual Organ Blood Volume of Cattle at Various Weights.

Wt (lb)	Total blood vol (ml/100 g)	ml blood/100 g (fresh wt)								
		Spleen	Lungs	Liver	Kidney	Pituitary	Adrenal	Heart	Pancreas	G. muscle
70	7.6	31.6	23.2	10.4	3.9	2.8	3.1	2.9	2.3	1.3
78	9.1	29.7	26.2	6.1	3.4	4.0	2.8	2.2	2.2	.8
83	8.5	32.6	25.8	7.2	3.7	3.4	3.2	2.7	3.1	1.0
Avg 77	8.4	31.3	25.1	7.9	3.7	3.4	3.0	2.6	2.5	.96
$\pm 7.7^*$	$\pm .9$	± 1.7	± 1.8	± 2.5	$\pm .3$	$\pm .7$	$\pm .2$	$\pm .4$	$\pm .5$	$\pm .12$
800	5.6	22.1	23.4	9.5	7.0	4.6	3.9	4.4	4.5	.7
758	5.8	39.3	18.2	6.0	6.9	4.3	3.5	3.0	3.2	.9
834	5.7	39.4	18.2	7.3	5.9	3.7	3.8	3.4	2.8	1.0
992	5.4	37.0	21.5	8.0	6.6	5.8	4.1	3.9	2.9	1.3
Avg 846	5.6	34.5	20.3	7.7	6.6	4.6	3.8	3.7	3.3	1.0
± 115	$\pm 2.$	± 3.6	± 2.5	± 1.7	$\pm .5$	$\pm 1.$	$\pm .3$	$\pm .7$	$\pm .8$	$\pm .2$

* Mean $\pm$ S.D.

TABLE III. Residual Organ Blood Volume of Sheep at Various Weights.

	Wt (lb)	Total blood vol (ml/100 g)	ml blood/100 g (fresh wt)										
			Spleen	Lungs	Liver	Kidney	Heart	Pan- creas	Adrenal	Pitui- tary	Loin muscle	G. muscle	
Avg ± 3.5*	6	10.0	33.0	16.3	10.3	6.2	5.3	4.0	1.7	1.9	2.6	2.0	
	10	9.3	36.1	18.2	9.8	5.7	6.3	3.2	1.9	2.2	1.3	1.4	
	12	8.5	35.4	20.1	8.6	4.8	6.2	2.1	2.1	2.0	1.8	1.6	
	9	9.3	34.8	18.2	9.6	5.6	5.9	3.1	1.9	2.0	1.9	1.7	
	± 3.5*	± .9	± 1.8	± 2.2	± 1.	± .8	± .6	± 1.1	± .2	± .2	± .8	± .4	
	96	6.2	33.0	29.4	9.1	5.3	5.5	4.0	2.2	2.1	1.1	1.5	
	102	6.1	36.8	29.0	6.6	5.3	4.4	2.5	4.2	2.0	.9	.7	
	136	5.5	34.2	21.5	8.6	6.4	5.5	3.3	3.1	2.5	1.5	1.2	
Avg ± 24	111	5.9	34.7	26.6	8.1	5.4	5.1	3.3	3.2	2.2	1.2	1.1	
		± 24	± .4	± 2.2	± 4.7	± 1.5	± .6	± .6	± .9	± 1.2	± .3	± .4	± .5

* Mean ± S.D.

blood, but the differences between old and young cattle were not consistent nor sufficient for significance in this study.

Residual blood in sheep organs and tissue. The milliliters of blood per 100 g of fresh tissue are shown for old and young sheep in Table III. The trend followed essentially the same pattern as was indicated for cattle, and the milliliter percentage of residual blood closely paralleled that for organs and tissues of cattle. When consideration is given to the fact that one-third and one-fourth of the weight of the spleen and of the lungs respectively were due to blood itself, the significance of this dilution factor for the interpretation of tissue analysis values can be realized. Other tissue and organ blood values ranged from one to 10% on a fresh weight basis.

Residual blood in swine organs and tissue. Residual blood determinations were made in tissues and organs from 10 swine at different ages and weights as indicated in Table IV. The calculated milliliters of residual blood per 100 g of fresh tissue show the lungs to be highest, followed by the spleen, kidney, liver, adrenals, heart, pancreas, loin muscle and gastrocnemius muscle in that order. There appeared to be a general tendency toward a higher concentration of residual blood in organs and muscle of the younger animals.

Discussion. The isotope dilution procedure using P^{32} labeled red cells has been found convenient and reliable for the quantitative measurement of the residual blood in organs and tissues of farm animals. In radio-isotope investigations and specific kinetic

studies with blood-borne substances these values require emphasis. Because of the intimate relationship between the cells of the body and the blood, inherent errors in analysis may be encountered due to residual blood trapped in the tissues at the time of slaughter. For the most part, routine sampling is made from animals that have been bled out; therefore, total organ blood volume (6-8) was not considered a necessity in this study. It is pointed out that since the residual blood calculations are based upon total blood volume values, consideration should be given to some of the more important sources of error when the isotope dilution procedure is employed. These errors invariably leading to a high blood volume value include: a) loss of label from cells injected, b) non-quantitative injection of cells into the blood stream and, c) removal of label from circulating blood following administration. Every precaution was taken in this study to avoid or to correct these errors, and by management practices to avoid physiological changes known to affect the normal animal. Since whole organs could not always be analyzed conveniently, multiple samples were taken routinely from similar areas of the various organs and processed immediately. The percent of the total blood volume for individual organs showed considerable variation, but residual blood per gram within tissues was remarkably constant. There was a general tendency for a lower residual blood concentration in tissues and organs of older and heavier animals, and a decrease for muscle tissue with increased age

TABLE IV. Residual Organ Blood Volume of Swine at Various Weights.

Wt (lb)	Total blood vol (ml/100 g)	ml blood/100 g (fresh wt)							Loin muscle	G. muscle
		Lungs	Spleen	Kidney	Liver	Adrenal	Heart	Pancreas		
8	7.1	26.2	13.1	6.5	8.1	5.0	4.0	2.2	2.2	1.0
7	7.4	27.8	12.3	6.3	7.8	6.0	4.6	2.1	2.2	1.2
10	7.0	25.2	12.5	6.8	8.3	5.7	3.3	1.7	2.2	1.0
12	6.9	24.8	12.2	5.3	6.4	6.3	3.1	2.5	2.1	.9
Avg 9	7.1	26.0	12.5	6.0	7.7	5.8	3.8	2.1	2.2	1.0
± 2*	± .2	± 1.5	± .4	± .7	± .9	± .6	± .7	± .4	± .0	± .1
28	6.8	22.9	15.1	7.1	5.1	5.4	3.4	2.1	1.4	.79
32	6.5	28.2	16.3	7.5	5.9	5.4	3.1	2.3	1.9	.80
78	6.4	23.4	12.5	6.9	6.1	4.0	2.8	2.3	1.2	.82
Avg 46	6.6	25.0	14.6	7.2	5.7	5.0	3.1	2.2	1.5	.80
± 30	± .2	± 3.1	± 2.2	± .4	± .6	± .8	± .4	± .1	± .4	± .02
644	3.3	23.1	9.9	5.4	8.4	3.6	3.2	1.9	.92	.89
668	3.1	24.8	10.0	11.1	7.3	5.4	4.1	2.5	.90	.73
547	4.0	24.1	10.1	10.7	8.2	4.4	3.5	2.4	.92	.82
Avg 620	3.5	24.0	10.0	9.1	7.9	4.5	3.6	2.3	.91	.84
± 71	± .5	± 1.	± .1	± 3.4	± .6	± 1.1	± .5	± .4	± .01	± .08

* Mean ± S.D.

and weight. Little difference between species was noted except that the spleen, liver, pancreas and gastrocnemius muscle of cattle and sheep retained more blood than did those of swine. The lower concentration of blood in muscle noted especially in aged swine may well have been due to the increased fat content of these animals.

Summary. The residual blood in organs and tissue, as a function of age and weight, for cattle, sheep and swine sacrificed by conventional methods was determined by the isotope dilution procedure using P^{32} labeled homologous erythrocytes. Organ weights and total blood volume compared favorably with literature reports. Neither age, weight nor species appeared to influence significantly the ml of tissue residual blood/100 g fresh weight, except that the spleen, liver, pancreas, and gastrocnemius muscle of cattle and sheep retained more blood than did those tissues of swine. There was a general tendency for higher residual blood concentrations in organs and tissue of younger animals, but these findings were not consistent. Adrenals, heart, pancreas, kidney, liver, pituitary and muscle tissue retained less than ten ml of blood/100 g fresh weight, while lungs and spleen re-

tained more residual blood. It is suggested that these values may provide a basis for estimated corrections in quantitative investigations for distribution of blood-borne substances in farm animals.

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Effect of Insulin on Free Tumor Cells in Peritoneal Exudate and on Their Host.* (22161)

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Soon after discovery by Warburg(1) of the high rate of glycolysis in tumors, several attempts were made to inhibit growth of tumors by influencing their sugar metabolism. Thus, Silberstein *et al.*(2) reported delay or complete inhibition of tumor growth in tarred animals after insulin treatment. This inhibition of carcinogenesis by insulin was observed also under various experimental conditions by von Witzleben(3), Muenzner and Rupp(4) and by Risse and Poos (quoted by Bauer(5)). Moreover, Braunstein(6) recorded for diabetics with tumors, better glucose tolerance and decrease of blood sugar level following increase in tumor size. However, Bauer(5) could not detect any variations of sugar metabolism in cancer patients or any change in their condition by variations of sugar level. This discrepancy in results was perhaps responsible for the lack of further publication on this problem as a careful search of the literature did not reveal any recent data on insulin effect in tumor-bearing animals. However, some statistical research has been done on the association of cancer with diabetes suggesting a possible relationship between sugar metabolism and malignant growth(7, 8,9).

The method of free tumor cell culture in peritoneal fluid(10-12) provided a useful tool for quantitative estimation of the effect of various factors on tumor cell growth. The use of globin insulin assured more continuous and uniform changes in blood sugar level. This selection of technic and material enabled us to obtain some quantitative information about insulin action on tumor cells and on their hosts.

Material and methods. Tumors Sarcoma-

180 and Sarcoma-37 were grown by serial intraperitoneal transfers in CFW (Carworth Farms) mice. All normal mice treated with insulin were of the same strain and age. Doses of 10^6 to 10^7 tumor cells from peritoneal exudate were inoculated intraperitoneally into mice. In each experiment, 20 mice remained untreated and 20 received daily intramuscular injections of insulin doses (0.25 to 2 units). Peritoneal exudates were obtained for total and differential cell counts on 4th and 5th day of tumor cell growth by technic previously described(10). For 10 mice of each series the percentage of mitoses in tumor cells was recorded in addition to differential counts. Similar examinations were carried out on exudate induced in 20 control mice by injection of normal tissue (liver, spleen) suspensions. In other series, inoculated and normal mice were treated simultaneously with daily insulin injections for 5 days and their survival span was recorded.

Results. Results of total, differential, and calculated tumor cell counts in peritoneal exudates from untreated and insulin treated animals are recorded in Table I.

The data of Table I show that average concentration of tumor cells was not influenced significantly by 3 insulin doses of 0.5 unit (series IA). It was decreased markedly by doses of 1 or 2 units (series 1B, 1C, 2A, 3A, 4A, 5A, and 6A). This effect was due to decrease either of total exudate cell number (3A and 5A) or of the proportion of tumor cells (2A, 6A) or to both (1B, 1C, 4A). This effect could be prevented by injection of massive doses of glucose immediately after insulin: the same dose of glucose alone did not induce any variations in cellular formula of exudate. Thus both total number of cells and number of tumor cells are apparently stimulated in the presence of both insulin and glucose. Percentage of mitoses in tumor cells of controls was 9 to 19 (average 12) and in in-

* This investigation was supported by grants from the American Cancer Society, upon recommendation of the Committee on Growth, and from the National Cancer Institute, National Institutes of Health, Public Health Service.

TABLE I. Growth of Free Tumor Cells in the Peritoneal Cavity of the Mouse under Insulin Treatment.

Exp. No.	Tumor inoculated, i.p.	Treatment with insulin		Results (averages and variation extremes—)			
		Days	Units daily	Amt of peritoneal exudate	No. of cells (tumor & leukocytes)/mm ³	% tumor cells	Calculated No. tumor cells/mm ³
1 A	S- 37	3	.5	3+	118	46	55
				2+—3+	53—191	32—60	27— 88
B		3	1	2+	92	39	34
				+—2+	60—112	14—53	15— 49
C		3	2	2+	46	36	15
				+—2+	32— 77	18—53	5— 23
D		0	0	3+	123	52	55
				2+—3+	72—157	41—78	43— 79
2 A	S-180	4	2	2+	23	21	5
				+—3+	19— 28	11—26	3— 7
B		0	0	3+	26	53	16
				2+—3+	16— 27	23—75	4— 45
3 A	"	4	1	2+	146	42	63
				+—3+	56—213	34—57	25—113
B		0	0	3+	193	39	78
				2+—3+	48—320	30—47	14—134
4 A	"	5	1	+	69	59	43
				+—2+	51— 91	30—72	15— 65
B		0	0	3+	109	68	77
				2+—3+	75—142	57—80	44—114
5 A	"	3	2	2+	117	28	31
				+—3+	70—173	11—55	8— 53
B		0	0	3+	280	31	76
				2+—3+	189—419	16—68	39—129
6 A	S- 37	4	1	2+	139	17	25
				+—3+	88—180	11—28	10— 39
B		4	1	3+	194	37	68
	1 cc 20% glucose daily			2+—3+	135—317	19—58	35—102
C		4	0	3+	84	38	32
	1 cc 20% glucose daily			2+—3+	36—188	25—62	10— 68
D		0	0	3+	105	31	35
				2+	39—156	10—50	11— 56

20 mice in each group. For total numbers and differential counts, averages and extremes are indicated. Amount of exudate estimated* as proportionate to volume of fluid obtained by insertion of glass capillary in peritoneal wall: 3+ = .5 mm³ or more, 2+ = <.5 but >.1 mm³, + = <.1 mm³, 0 = no fluid.

* Reliability of this estimate was shown by comparison with the amount of exudate measured at autopsy.

sulin treated—0 to 11 (average 5). No significant variations (more than 10% of the average) in the leucocyte formula were noted in the exudate induced by normal tissue suspension.

Effect of insulin on host of tumor growth was estimated by comparing the life span in 3 groups of mice: 1) tumor-bearing and untreated, 2) tumor-bearing and insulin treated and 3) insulin treated normal mice. The results are reported in Table II.

It appears from Table II that mice of

group 2 died earlier than in control groups 1 and 3. Administration of glucose after insulin decreased mortality rate in group 3, but not in group 2. Doses of 0.25 unit of insulin were insufficient to influence survival span of mice.

Analogous experiments were carried out in mice bearing subcutaneous tumors with similar findings but with a wider range of variations.

Discussion. Decrease in number of free tumor cells in the exudate after insulin treat-

TABLE II. Survival of New and Tumor-Bearing Mice Treated with High Doses of Globin Insulin.

Days after 1st insulin inj.	Mice with tumor growth					Mice without tumor growth (new mice)			
	Treated daily with requisite doses (units) of insulin								
	Group 1	Group 2				Group 3			
	Untreated controls (tu- mor growth, no insulin)	Tumor growth and insulin treatment				Insulin treatment, no tumor growth			
		.25	.5	1-2	1 + glucose	.25	.5	1	1 + glucose
3	20	20	18	15	20	20	17	15	18
5	19	18	12	13	12	20	15	13	16
7	14	16	7	8	7	20	12	11	14
10	11	9	2	1	2	20	12	11	14

Mice were inoculated intraperitoneally with 10⁶ S-180 cells 4 hr before 1st insulin injection. Insulin given 5 successive days. 20 animals in each series. Dose of glucose: 1 cc of 20% solution. Number of surviving mice indicated.

ment could neither be interpreted as effect of dilution, since volume of exudate was reduced (Table I), nor as increase in proportion of leucocytes, because insulin did not modify the cellular formula of exudate in controls. Moreover, decrease in percentage of mitoses indicated the effect of some factor in the peritoneal fluid on the floating tumor cells. This factor was either insulin or some metabolic product of hypoglycemia, since glucose prevented cell damage.

Growth inhibition of tumor cells by insulin excluded obviously the possibility of incriminating malignant growth for early death of their hosts; on the other hand, comparison with results in insulin treated normal mice pointed out that sensitivity to insulin was increased by occurrence of malignant growth. Since the lethal effect of high insulin doses was partly reversible by glucose in normal mice and irreversible in tumor hosts, it may be presumed that in the latter some vitally important tissues were damaged by hypoglycemia and its products, or by insulin itself; for instance, it may be presumed that insulin increased cell permeability(13,14) for some toxic metabolites of malignant growth. Since combined treatment with insulin and glucose induced far greater effect on exudate and tumor cells than simple reversal of insulin action, a further study of this phenomenon may offer new insight in our problem.

Summary and conclusions. CFW mice were inoculated i.p. with S-180 or S-37 cell suspensions and treated with 3 daily i.m. in-

jections of globin insulin (0.5 to 2 units). On the 4th or 5th day, specimens of peritoneal exudate were withdrawn from these mice and from untreated controls; the number of tumor cells per cmm and the percentage of their mitoses were consistently lower in insulin treated animals; this effect of insulin could be prevented by repeated injections of glucose (1 cc of 20% solution). In other experiments, the survival span was recorded for insulin treated tumor-bearing animals, their untreated controls and insulin treated normal mice. The first group showed the shortest survival which could not be extended by injections of glucose. It is concluded that growth of free tumor cells in the exudate was reversibly inhibited by hypoglycemia, its metabolic products or by the direct effect of insulin on tumor cells (permeability?) and that the same factors (or some of them) provided additional stress to some vitally important tissues damaged by tumor growth.

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Antifibromatogenic Activity of 19-Nor- α -ethinyltestosterone in the Guinea Pig.* (22162)

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Both the progestational and antifibromatogenic potencies of 19-nor-progesterone (19-nor-P) are greatly superior to those of progesterone (P)(1,2). Likewise, the progestational potency of (19-nor- α -ethinyltestosterone (19-nor-ET)(3) has been found to be 5 times that of α -ethinyltestosterone (ET) (4). ET also is antifibromatogenic though about 15 times less than P(5,6). Because of the clinical importance of 19-nor-ET which is active by mouth(4) its antifibromatogenic activity was studied.

Methods previously described(2) were used. Because of slow absorption of ET (0.2 to 0.4 μ g/sq mm of the pellet) 4 to 8 pellets were implanted subcutaneously. Absorption rate of 19-nor-ET was 1.6 μ g/sq mm. Absorption rate of 19-nor-P also was greater than that of P (7 against 3 μ g/sq mm(2,5,7).

Results with ET and 19-nor-ET are summarized in Table I. No prevention of estrogen-induced abdominal fibroids was obtained with the quantities of ET used. On the contrary, with the same quantities of 19-nor-ET the fibrous reaction was greatly diminished; the same occurred even with as little as 50 to 70 μ g/day (see individual values in parentheses). With ET values of 4 to 7 may still occur with 150 to 210 μ g/day(5). Thus the antifibromatogenic potency of 19-nor-ET is probably more than 4 times that of ET. It is noteworthy that the increase from ET to 19-nor-ET is of the same quantitative order as from P to 19-nor-P(2). However, 19-nor-ET is far less potent than 19-nor-P and even than P.

There was also a very pronounced increase in prevention of abnormal uterine weights

TABLE I. Antifibromatogenic and Antihysterotropic Activities of ET and 19-nor-ET in Castrated Female Guinea-Pigs with Subcutaneously Implanted Pellets of Estradiol (Absorption: 47 to 50 μ g/day). Duration of experiment: 3 mo. For classification of results see (6,8).

Steroid tested—		No. of animals	Fibrous tumoral effect*	Q ₂₋₃ †	Uterine wt, g
Compound	μ g/day				
ET	71 (35-127)‡	10	6 $\pm$.9	1.9	6.0 (2.7-8.7)
19-nor-ET	80 (49-126)§	14	2 $\pm$.2	.3	2.6 (1.5-4.0)

* Arbitrary values for number and size of tumors, ranging from zero to 12(6).
† " " " large tumors only, per animal (8).
‡ 4 to 8 pellets.
§ 1/2 to 1 pellet.
|| Individual values: 2-2.5-3-4-5.5-6.5-7.5-7.5-8.5-11.
" " " : (1.5-1.5)-1.5-1.5-1.5-1.5-1.5-(2-2)-2-2.5-(3-3)-3. In parentheses: animals receiving only 50-70 μ g/day of 19-nor-ET.

* Cordial thanks are due to: Professor Carl Djerassi, Wayne Univ., Detroit, for 19-nor-ET; Messrs. Ciba, Basel, for ET; Syntex, S. A., Mexico, for a generous supply of estradiol.

which dropped from 6 g with ET to 2.6 g with 19-nor-ET.

Summary. The antifibromatogenic potency of 19-nor-ET is about 4 times that of ET. 19-nor-ET is also greatly superior to ET in antihysterotrophic potency, *i.e.*, in preventing uterine increase due to estrogen.

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Effects of Hallucinogenic and Tranquilizing Drugs on Serotonin Evoked Uterine Contractions. (22163)

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Evidence has been presented to support the hypothesis that serotonin (5 hydroxytryptamine) which has been found in extracts of mammalian brain(1,2) plays a role in the physiology of the nervous system of invertebrates(3,4). While many physiological properties of serotonin have been characterized (5), its action on the central nervous system has not been defined. Shaw and Woolley(6) think that serotonin injected in the mouse cannot reach the brain. Since the hallucinogen, d-lysergic acid diethylamide (LSD), has been shown to antagonize the effects of serotonin, both *in vivo*(7,8) and *in vitro*(9), it has been suggested that serotonin antagonism may be important in the mechanism of LSD induced psychosis(9). However, mescaline and bufotenine(10) which are also hallucinogenic, do not antagonize serotonin activity *in vitro*(9,5). Furthermore, while many lysergic acid derivatives are not hallucinogenic, they retain the ability to antagonize serotonin-induced uterine contractions(7).

Methods. In the present experiments we used the uterus of spayed rats brought into oestrus by the injection of ovarian hormone.

The rat uterus was suspended (at 28.5-29.5°C) in aerated de Jalon fluid (NaCl 9.0 g; KCl 0.42 g; CaCl₂ 0.06 g; NaHCO₃ 0.5 g; glucose 0.5 g/l). We studied the reactivity of the organ to serotonin creatinine sulfate, acetylcholine (ACh) and oxytocin before and after treatment with Frenquel (alpha-4-piperidyl diphenyl carbinol hydrochloride), chlorpromazine (10-(3-diethylaminopropyl), 2-chlorophenothiazine hydrochloride), reserpine, desmethoxy reserpine, Lergigan (N (2-dimethylamino-2-methyl-1-ethyl) phenothiazine hydrochloride), mescaline (3,4,5 trimethoxyphenyl ethylamine sulfate) and LSD (d-lysergic acid diethylamide). To make a comparative evaluation of the antagonistic powers as well as to compare the specificity of each drug tested, we determined the uterine reactivity to a series of increasing concentrations of contractile agents, (serotonin, ACh and oxytocin) starting from a minimally active concentration then increasing it until the maximal uterine contraction was reached. Each dose was given 3 times. We accepted, as the stabilized value, the mean height of contraction when the variation between the 3 contractions was not greater than 10%. Then we again tested the reactivity of the uterus to the contractile agents after the hallucinogenic or

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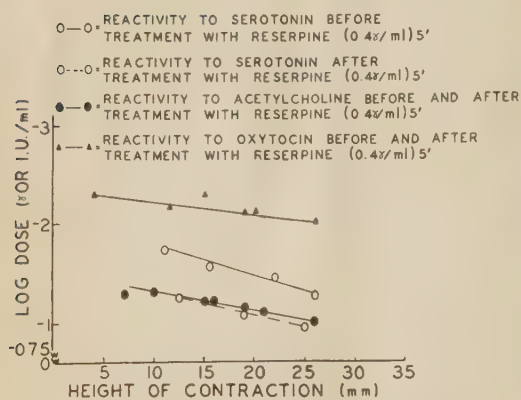


FIG. 1. Diminished contractility of uterus to serotonin after reserpine is contrasted to constancy of response to ACh and oxytocin. The figure discloses that to obtain an equal degree of activity after reserpine it is necessary to use a higher concentration of serotonin.

tranquilizing drug (Frenquel, chlorpromazine, reserpine, Lergigan, mescaline and LSD) had been left in contact with the organ for 3 to 5 minutes.

Results. The tranquilizing agents (Frenquel, reserpine, deserpidine and chlorpromazine) antagonize the serotonin-induced uterine contractions while in the same doses they display no antagonism either toward ACh or oxytocin. Lergigan and Meratran, similar in their chemical structure to chlorpromazine and Frenquel, respectively, do not display anti-serotonin activity in the concentrations at which they antagonize ACh. Successive washing completely reversed the antagonistic effects of Frenquel and of chlorpromazine after intervals of 30 and 60 minutes respectively. With reserpine and its analogue (both in concentration between 0.4 to 1 mg/l), the antagonism was long lasting; the uterine contractility being restored to normal values after 240 minutes.

Fig. 1 shows the uterine response to serotonin, ACh, and oxytocin before and after treatment with reserpine (0.4 mg/l) for 5 minutes. The negative log of concentration (γ/cc) of the contracting agent (serotonin, ACh and oxytocin I.U./cc) was placed on the ordinate axis, and the mean height of contraction (in mm) attained with each dose on the abscissae. Thus we obtained a graphic representation of the reactivity of the uterus

to the contracting agent. No changes were observed in the response to oxytocin before and after treatment with reserpine. The graphical expression of uterine reactivity to ACh both before and after treatment practically coincides showing that no changes were induced by the drug. The open circles represent the uterine reaction to serotonin; the upper line showing the pretreatment reactivity, and the lower one indicating the decrease in sensitivity following treatment. The distance between these two lines represents the degree of inhibition exerted by reserpine on the serotonin-induced uterine contractions. Chlorpromazine (0.1 γ/cc for 3 minutes) and Frenquel (0.8 γ/cc for 3 minutes) increased the minimal active dose of serotonin from 12 γ/l to 60 γ/l and from 14 γ/l to 38 γ/l respectively.

Mescaline (Fig. 2) in concentration of 100 γ/l , facilitated the serotonin-induced contractions of the rat uterus. The increase of the serotonin contractions induced by mescaline is a reversible phenomenon. Mescaline alone in concentrations higher (over 0.4 mg/l) than that used in the above experiment evoked a uterine contraction. The contractile effect of mescaline was not modified by 240 γ/l of atropine sulfate. This concentration usually antagonized ACh-induced contractions of the rat uterus.

With LSD we were able to confirm the results of Gaddum(9), Cerletti(11), and Erspamer(5). As a matter of fact, using the comparatively high concentration of LSD of 1 γ/l (left in contact with the uterus for 5 minutes), we obtained a clear inhibition of the serotonin or mescaline-induced contraction,

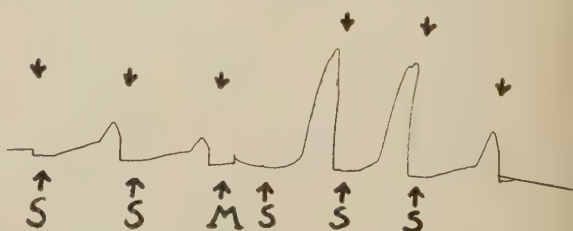


FIG. 2. Mescaline (100 γ/l) produces a transitory augmentation of minimal contraction evoked by serotonin (2 γ/l). M and S indicate treatment of uterus with mescaline and serotonin respectively. In the concentration employed in this experiment mescaline by itself cannot cause a contraction.

while the activity of ACh was not affected. In contrast by using minimal effective doses of serotonin, we observed that LSD in low amounts (0.05 to 0.2 γ /l) left in contact with the uterus for 3 minutes increases the uterine response both to serotonin and mescaline. Since the action of LSD is long lasting, with the use of successive doses a summation of effect may occur. Thus inhibitory concentrations can be easily reached which prevent the demonstration of the facilitating action of LSD in low concentration.

Discussion. We were able to demonstrate that reserpine, Frenquel, and chlorpromazine are antagonists of serotonin activity in the rat uterus. Meratran and Lergigan, similar chemically to Frenquel and chlorpromazine respectively, do not exert a tranquilizing action in patients, and do not antagonize the serotonin-induced uterine contraction specifically. Mescaline and LSD, on the other hand, show a great affinity for serotonin receptors and under our experimental conditions, could be considered synergistic with serotonin. If an analogy is possible between the receptors in smooth muscle and the serotonin receptors in the brain, tranquilizing action seems to be associated with serotonin inhibiting properties while the hallucinogenic effect may be related to the facilitation of serotonin.

Summary. Tranquilizing drugs: Frenquel (0.8 mg/l), chlorpromazine (0.1 mg/l) and reserpine (0.4 mg/l), antagonize serotonin-induced uterine contractions, though they do

not affect sensitivity of the uterus of the spayed rat either to ACh or oxytocin. The period of antagonism is longer lasting with reserpine than with chlorpromazine and is shortest with Frenquel. Mescaline (0.1 mg/l) causes a facilitation of serotonin activity, and in highest amounts causes a uterine contraction by itself. However, it is not antagonized by atropine (0.24 mg/l). A relatively high concentration of LSD (1 γ /l) antagonizes both serotonin and mescaline-induced uterine activity. In contrast low concentrations of LSD (0.05 to 0.2 γ /l) facilitate the effects of serotonin and of mescaline on the rat uterus.

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Occurrence in Plasma of an Extractable Lipid Mobilizer (LM)* (22164)

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Plasma from rats treated with cortisone, exposed to cold or subjected to the nephrotic syndrome prevented the delactescent action of heparin clearing factor *in vitro*(1). Hypercholesterolemia which was independent of adrenals or renal lesions was observed in rabbits prepared for the Schwartzman reaction and represented another example of the effect on lipids by non-specific stimuli(2). These observations suggested that concentration of a lipid mobilizer (LM) in the plasma could be increased either by cortisone or stress. In this paper we are reporting separation of such a substance from plasma of untreated and cortisone treated animals, also data which demonstrate the effect of LM on plasma lipids, food intake and body weight.

Materials and methods. Blood was collected in 0.1 M sodium citrate (1:9 whole blood) and centrifuged. The decanted plasma (2 ml/rat; 5 ml/human; 150 ml/dog) was dialyzed through Viscose Nu-Jax® casing against an equal volume of demineralized water for 6 hours at 25°C with constant agitation. Bulk lots were prepared from several

liters of horse plasma under conditions that minimized bacterial and pyrogen contamination. The horse blood was collected aseptically and stored at 5°C for 72 hours to permit settling of the cells. The viscose casing was washed with 0.5% phenol and sterile, pyrogen-free demineralized water. The dialysis was at 25°C for 72 hours against sterile, pyrogen-free demineralized water in closed sterile containers. The dialyzate was concentrated at 75°C and 0.2 mm Hg pressure for 72 hours and freeze-dried for a similar time. The final product was sterile and did not produce fever in the animals used in the lipemia experiments. All animals were fasted 24 hours before blood samples were taken or before cortisone was administered. In the latter group, blood samples were obtained 4 hours after the administration of the cortisone. The LD₅₀ of the dry extract was determined in Manor Farm All-Purpose mice, Wistar rats, guinea pigs, albino rabbits and mongrel dogs. A dose response curve was determined in normal Wistar rats and hypophysectomized albino rats. The effect of chronic administration of large

TABLE I. LM Activity of Plasma Dialyzates Prepared from Untreated and Cortisone-Injected Animals.

Species	Treatment†	No. of animals	No. of dialyzates showing LM activity	Hyperlipemic dose of dialyzate, mg/kg, I.V. in rats*
Human	None	10	10	285
Horse	None	4	4	300
	Cortisone	6	6	1
Dog	None	8	3	325
	Cortisone	5	5	1.85
Rat	None	10	10	300
	Cortisone	10	10	0.95
	Hypophysectomy Cortisone	10	0	>500

* Log spaced doses (10 rats/dose for each dialyzate; at least 100 rats per dialyzate).

† 5 mg/kg intramuscular.

* We are grateful to W. Bechmann, E. Livingston and G. Nash for their valuable assistance in the animal studies; to Doctors H. E. Alburn and H. Tint for guidance in preparation of the dialyzate; to Doctors B. Scott Fritz, J. Brown and B. Criley for

preparing the large batches of sterile dialyzate from horses; to Doctors J. W. Gofman and B. Shore for supplying the DFP; and to Doctor G. H. Ellis for the infra-red determinations.

SENSITIVITY OF RATS TO HYPERLIPEMIC
ACTION OF LM(Wy 991)
(DOSE - RESPONSES)

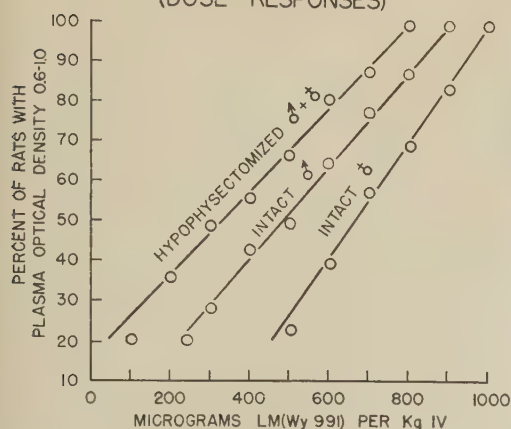


FIG. 1. Sensitivity of rats to action of LM.

doses of LM was studied in rabbits and dogs. At weekly intervals venous blood was analyzed for lipid phosphorus by the method of Fiske and SubbaRow(3), total fatty acids by the method of Stoddard and Dury(4) and total cholesterol by the Sperry-Schoenheimer technic(5). Optical density of the plasma was measured in a model B Beckman spectrophotometer.

Results. The LM activity of various plasma dialyzates when injected into rats is shown in Table I. Of the 63 dialyzates injected 15 failed to exhibit LM activity; these included 5 from untreated dogs and 10 from hypophysectomized rats. On the basis of mg of dialyzate/kg of body weight cortisone treatment caused an increase of approximately 300-fold in LM activity but on the basis of volume of original plasma the increase was approximately 1000-fold. LM activity was considered to be present when the optical density of the plasma of the in-

jected rats increased from the initial 0.01 to at least 0.60. Plasmas exhibiting lipemia by this method also had at least a 50% elevation of total and esterified cholesterol, total fatty acids and lipid phosphorus. The dose necessary to elicit LM activity was 1/50 the LD₅₀ or less. The intravenous LD₅₀ of the dialyzates prepared from cortisonized animals calculated by the method of Bliss was as follows: (mg/kg) rats 48; guinea pigs 45; rabbits greater than 100; and dogs greater than 75. The injection of cortisone into hypophysectomized rats failed to cause the release of significant amounts of LM.

The dose response curves illustrated in Fig. 1 show that female rats were more resistant than males to the hyperlipemic action of LM and that intact rats were more resistant than hypophysectomized ones. In the hypophysectomized group females were as sensitive as the males. Each point on the curves represents 10 rats. The curves are qualitatively similar (no deviation from parallelism: $X^2 = 0.3$; $P > 0.5$) but differ quantitatively ($F = 13.4$; $P < 0.05$). The hyperlipemia resulting from injection of the ED₁₀₀ (1 mg/kg) persisted for at least 6 hours and that from 60 mg/kg for at least 7 days. The degree of hyperlipemia following injection of a single dose of LM is shown in Table II. The error in the methods employed was smaller than the standard deviations. There was a prompt 2-fold increase of all plasma lipid components in the species tested. The hyperlipemia was invariably associated with increased optical density except in humans where there was no correlation. The ED₁₀₀ in rats by intramuscular injection was 5 mg/kg. Intragastric administration of 100 mg/kg failed to produce hyperlipemia in rats. In conjunction

TABLE II. Mean Concentration (mg %) of Lipids in Plasma of Animals 1 Hr after Injection of LM.

Animal	No.	mg/kg	Total Ch		FA		LP		OD	
			Initial	1 hr	Initial	1 hr	Initial	1 hr	Initial	1 hr
Mice	100	.5	53	174	148	450	5	12	.05	.90
Rats	475	.10	72	190	155	480	5	13	.04	.86
Guinea pigs	100	.50	66	185	160	440	5	12	.06	.80
Rabbits	40	.50	50	172	200	420	5	11	.05	.86
Dogs	20	1.	86	168	209	430	6	11	.05	.92

Ch = Cholesterol; FA = Fatty acids; LP = Lipid phosphorus; OD = Optical density.

TABLE III. Effect of LM (Mean Values mg %) on Plasma Levels of Lipids in 6 Dogs (60 mg Dialyzate/kg I.V. Once Weekly).

	Week of treatment									
	0	1	2	3	4	5	6	7*	8	9
Total Ch	83	165	190	220	245	240	210	240	202	189
Ester Ch	58	110	118	134	150	142	135	142	122	110
Total FA	212	440	520	680	590	540	606	630	420	250
Lipid P	7	12	12	14	13	13	12	13	10	8
Food taken daily (g)	265	370	540	550	550	552	550	550	500	450
Body wt (kg)	6	6	5	4	4	4	4	5	6	9

* LM discontinued.

Ch = Cholesterol; FA = Fatty acids.

with Dr. William Steiger, Dept. of Medicine, Temple University, 5 human subjects were injected intravenously with 0.25 to 1 mg dialyzate/kg. All responded with elevated plasma concentrations of cholesterol, fatty acids and lipid P of the same magnitude as the other species tested. None of the humans developed chills, fever, or other signs of toxic reactions.

Repeated single weekly injections of large doses of dialyzate containing LM in dogs and rabbits resulted in sustained hyperlipemia in which all elements of the plasma lipids were still elevated at least 2-fold 7 days following injection (Tables III and IV). The hyperlipemic animals developed marked hyperphagia in which food intake was 2-3 times that of control animals. The hyperphagic animals did not gain in body weight as rapidly as the controls and in some instances there was loss of body weight amounting to 33% of the initial. Discontinuing the administration of LM resulted in subsidence of the hyperlipemia and hyperphagia with an accompanying gain in body weight. Gross examination of dogs autopsied after 4 and 5

weeks of LM administration showed almost complete atrophy of the perirenal, omental, mesenteric and spermatic cord fat depots and marked depletion of subcutaneous abdominal fat. The livers were not fatty nor was there evidence of lipid deposition in other viscera or in the blood vessels.

The anti-clearing (CFI) action of a dialyzate prepared from horses injected with cortisone is illustrated in Fig. 2. The *in vitro* method(1) utilized lipemic plasma and plasma from heparinized animals.

Discussion. The LM activity of the plasma dialyzate prepared from cortisonized animals was probably due to a mediator released by the injected cortisone. The dialyzate contained no cortisone or other steroids when it was subjected to infra-red analysis or to the Porter Silber and Liebermann-Burchard reactions. Cortisone requires conditions for dialysis which differ from those we employed(9). Assuming that all of the injected cortisone was absorbed and remained concentrated in the plasma and then entered the dialyzate, the amount injected was less than 10 μ g/kg of body weight. Only 20% of rats receiving 5 mg of cortisone/kg of body weight developed hyperlipemia in contrast to the 100% receiving 1 mg of dialyzate/kg of body weight. Finally, plasma dialyzate from hypophysectomized rats injected with cortisone had no LM activity.

LM differed from the preparation of protamine available to us in being dialyzable and heat stable. Although LM and protamine(6) are CFIs *in vitro* and produce hyperlipemia when injected into animals other inhibitors such as diisopropylfluorophosphate (DFP)

TABLE IV. Effect of LM (Mean Values mg %) on Plasma Levels of Lipids in 20 Rabbits (60 mg Dialyzate/kg I.V. Once Weekly).

	Week of treatment					
	0	1	2	4	5	6
Total Ch	72	150	200	290	350	350
Total fatty acid	200	325	400	490	580	575
Lipid P	6	9	13	13	14	13
Food taken daily (g)	190	300	575	800	950	1000
Body wt (kg)	2.4	2.7	2.5	2.6	2.8	2.7

Ch = Cholesterol; FA = Fatty acid.

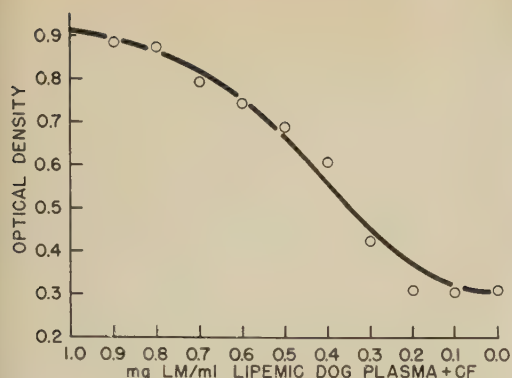


FIG. 2. CFI activity *in vitro* of LM plasma dialyzate vs. heparin clearing factor.

(7), and sodium cholate(8) failed to do so when administered in non-convulsant doses. It appears therefore that the hyperlipemic action of the dialyzate may not be related to its CFI action. The injection of LM into hypophysectomized rats produced hyperlipemia, whereas the injection of protamine failed to do so. Injection of 5 mg arginine/kg did not produce hyperlipemia in rats; larger doses resulted in fatality.

Growth hormone prepared from pituitary glands has been reported to be an active lipid mobilizer(10). LM differs from such preparations in being dialyzable, heat stable and in failing to promote growth in the presence of hyperphagia.

LM appears to resemble the factor extractable from the anterior pituitary which is free from growth-promoting or ACTH properties but is capable of mobilizing lipids(11). Several peptide fragments derived from the decomposition of the adipokinetic hormone are also capable of causing deposition of lipids in the liver(12). Our material, although dialyzable, is not a fragment produced by extraction procedures since the dialyzate without further treatment was capable of producing intense hyperlipemia.

Summary. 1. Freeze dried dialyzates of plasma prepared from dogs, rats and horses receiving cortisone contained a lipid mobilizer (LM) which markedly elevated plasma levels of free and esterified cholesterol, lipid P

and total fatty acids when administered to fasted mice, rats, rabbits, guinea pigs, dogs and humans by intramuscular or intravenous injection. LM was not active by the oral route. The dialyzate also inhibited *in vitro* the delactescing action of heparin clearing factor. Plasma dialyzates prepared from untreated humans, horses, dogs and rats were 1/1000 as potent. 2. Cortisone injection failed to release LM in hypophysectomized rats. 3. LM activity of the dialyzate is not due to the same constituent responsible for CFI activity. 4. Hyperlipemia resulting from a single large dose persisted for at least one week in rabbits and dogs. 5. Repeated large doses of LM resulted in sustained and progressive hyperlipemia, hyperphagia and either loss in body weight or failure to gain. 6. Marked depletion of perirenal, mesenteric, omental, spermatic cord and subcutaneous fat were observed in dogs receiving weekly injections of LM.

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Effect of Growth Hormone on Sulfate Tm, Urea Clearance and Fasting Blood Glucose.*† (22165)

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Growth hormone has a variety of actions in animals(1): it promotes nitrogen storage; it produces diabetes mellitus; and more recently it has been shown to exert a renotropic action in increasing the reduced filtration rate, renal plasma flow and Tm_{PAH} of hypophysectomized dogs(2) and increasing these functions in intact dogs after more prolonged treatment(3).

During a recent study of the effect of diet and hormones on sulfate Tm, we observed that cortisone, as well as a low-protein diet, increased this value(4). Since cortisone tends to accelerate protein catabolism, and because of the intimate relationship of sulfate to protein metabolism, we have considered the possibility that sulfate Tm is influenced by a change in direction of growth hormone, as a protein-anabolic agent, on sulfate Tm. We have measured in addition the changes induced in glomerular filtration rate, renal blood flow and fasting blood glucose.

Methods. Observations have been made on 5 trained, unanesthetized female dogs maintained on laboratory diet which supplied approximately 60 to 100 g of protein daily. They were fasted 16 to 20 hours before an experiment and to insure hydration were given 700 to 1000 cc of water by stomach tube about an hour before the experiment. Glomerular filtration rate and (effective) renal plasma flow were measured, respectively, by clearances of creatinine and PAH which were administered intravenously by means of a constant-rate infusion pump. Na_2SO_4 was incorporated in the infusion fluid to give a concentration of 1% and sulfate Tm was deter-

mined at load/T ratios of 1.5 to 2. At low plasma levels of PAH this substance does not interfere with sulfate reabsorption(5). Urea clearance (at least three 15-minute periods) was determined at constant or decreasing urine flows about 2 cc/minute. Creatinine was determined by the method of Bonsnes and Taussky(6); PAH by the method of Smith *et al.*(7); sulfate by the method of Power and Wakefield(8); urea by the method of Seligson and Seligson(9); and whole blood glucose by the Nelson-Somogyi method(10). Three growth hormone preparations were used.§ Two preparations, A and C, were made by the Raben-Westermeyer method(11), and preparation B by the Wilhelmi method(12). Preparation A was in powder form and was dissolved in distilled water acidified to pH 3 with 0.1 N HCl immediately before use. Preparation B (Armour Lots Nos. R 491131 B, R 527094 B, R 491092 B) was supplied as a powder in sterile vials and was dissolved in water before use. Preparation C (G-25-32) was in sterile solution kept frozen before use. Growth hormone was given in single intramuscular injection in the afternoon on successive days, and measurements of renal function were made in the morning after the third injection. In a few instances the hormone was injected intravenously to determine its acute effect over the following 60 minutes.

Results. 3 mg/kg/day for 3 days of preparation B increased sulfate Tm 200% and 68%, respectively, in 2 out of 3 dogs (Table I). (The third dog, No. 2, previously had shown an increase when given a low-protein diet for the first time, but not when placed on a low-protein diet a second time.) Plasma urea concentration and

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TABLE I. Effect of Growth Hormone in 4 Normal Dogs.

Dog	Wt, kg	Hct, %	Conditions	GFR,* cc/min.	RPF,* cc/min.	Sulfate Tm,* μ M/min./kg	Plasma urea, mg/100 cc	Urea excretion, mg/min.	Urea clearance,* cc/min.	Blood glucose, mg/100 cc
2	16.3	38	Md††	61.9	238	8.3	32.0	12.9	40.5	89
	16.8	44	3 mg B†	78.2	307	8.0	15.0	7.8	50.6	137
5	12.2	44	Md	69.8	205	8.2	48.0	18.4	42.4	103
	11.7	44	3 mg B	86.0	267	24.5	11.1	6.6	59.3	102
4	12.0	37	Md	62.0	194	9.3	21.0	8.6	41.3	97
	12.5	38	3 mg B	80.7	240	15.6	6.7	4.0	60.1	146
	13.4	41	Md $\times$ 6 days	73.3	254	7.7	45.0	20.6	45.8	106
1	8.5	39	Md	32.5	92	9.1	32.0	8.6	26.7	93
	9.0	35	5 mg Ct‡§	47.5	150	14.5	23.7	7.0	29.5	119

* Values for GFR, RPF, sulfate Tm and urea clearance are average of 3 periods.

† Md = mixed diet; B = B/kg/day $\times$ 3 days; C = C/kg/day $\times$ 3 days.

‡ This dog had just received 3 mg A/kg/day for 3 days without effect on renal function or blood sugar.

§ Lactation occurred in this dog with a dose of 3 mg/kg/day on third day, and again in a second trial with 5 mg/kg/day of preparation C after 3 days.

rate of urea excretion decreased in all 3 dogs. Plasma concentration decreased more than urea excretion in consequence of an increase in urea clearance of 25 to 47% over control values.

The filtration rate and effective renal plasma flow were increased by an average of 26% and 28%, respectively. In one dog, No. 5, blood glucose did not increase even though renal function was increased.

The two Raben preparations were less active than the Wilhelmi preparation in all respects. In dog No. 1, 3 mg/kg/day of preparation C for 3 days had very little effect on renal function or blood glucose concentration. Subsequently 5 mg/kg increased filtration rate and renal plasma flow but the effect on urea clearance and protein metabolism was slight (last experiment, Table I).

In experiments not reported here it was observed that 24-hour urine output, along with water intake, was often increased 3- or 4-fold after administration of preparation B. Moreover, during acute experiments listed in Table I, the animals treated with preparation B (in contrast to those treated with A or C) responded to a water load with a greater urine flow, resulting in greater recovery of administered water at the end of 2 hours (Table II). In view of the effect of water diuresis on urea clearance and urea excretion(13,14) we explored the possibility that diuresis in treated animals had increased urea clearance. This figure was therefore compared before and after hormone administration in the same dog at similar creatinine U/P ratios (Fig. 1). In each case the urea clearance was greater, at the same creatinine U/P ratio, after treatment, showing that diuresis was not an important factor.

In contrast to the effect of repeated administration of growth hormone intramuscularly, a single intravenous injection appeared to have no immediate effect. In 6 experiments on 4 dogs, a dose of 3 mg/kg of preparation B injected intravenously after 3 or 4 control periods produced no change in plasma urea concentration, urea excretion, filtration rate, renal plasma flow or sulfate Tm in the following 60 minutes.

TABLE II. Effect of Growth Hormone on Excretion of a Water Load in Normal Dogs.

Dog No.	Conditions	% water load excreted		GFR, cc/min.	Min creatinine U/P ratio
		1 hr	2 hr		
2	Md*	7	28	61.9	8.8
	3 mg B	17	62	78.2	8.2
4	Md	18	47	62.0	11.1
	3 mg B	19	70	80.7	9.8
1	Md	19	36	32.5	7.2
	5 mg C†	17	37	47.5	9.1
1	Low protein diet × 10 days	6	46	35.2	10.4
	Low protein diet and 1.3 mg B	35	99	49.7	9.0

* Md = Mixed diet; B = B/kg/day × 3 days; C = C/kg/day × 3 days.

† Dog lactating.

Discussion. Though the overall action of growth hormone is to spare protein(1), as exemplified by decrease in urea excretion in our experiments, the hormone tends to increase urea excretion at the expense of body urea in consequence of increased filtration rate. If protein metabolism remains constant, an increase in urea clearance will transiently increase excretion of urea until equilibrium is attained at a lower plasma concentration. In addition, since urea clearance increases with

increasing urine flow(13), an increase in water intake and urine flow will further increase urea excretion at a constant filtration rate.

Cortisone and growth hormone have, in general, opposite effects on protein metabolism, the first promoting protein catabolism, the second promoting anabolism; but both hormones increase sulfate Tm. Hence the effects on protein metabolism and on sulfate Tm are probably not directly related. The possibility remains, however, that some intermediate change in protein metabolism which may be induced by both hormones, such as, for example, a decrease in plasma level of one or more particular amino acids(15), induces changes in sulfate Tm.

It appears that renotropic action of growth hormone is one of its earliest observable effects. To induce diabetes mellitus, with similarly prepared extracts, either slightly larger doses have been used or injections have been continued for longer periods(16,17). Finally, it should be noted that the Wilhelmi preparation, which in our hands had greater renotropic and nitrogen-sparing effects than the Raben preparations, has been reported by Houssay and Rodriguez(17) to be more diabetogenic.

Summary. Growth hormone increased filtration rate, renal plasma flow, and urea clearance in 4 normal dogs, and sulfate Tm in 3 of these dogs, within 3 days. In 3 of the 4 dogs the fasting blood glucose concentration was elevated. Urea excretion (in short clearance periods) was decreased, but owing to the increased urea clearance, the plasma urea con-

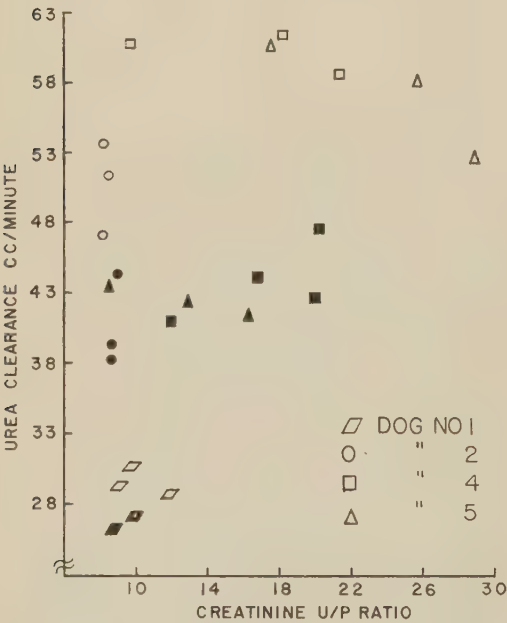


FIG. 1. Comparisons of urea clearances at similar creatinine U/P ratios before and after growth hormone in 4 dogs. Closed symbols, before treatment; open symbols, after treatment. Each symbol is a single clearance period. All observations were made at constant or decreasing urine flows.

centration decreased relatively more than urea excretion.

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Evidence for Participation of Vit. B₆ in Glutamine- α -Keto Acid Transamination-Deamidation Reaction. (22166)

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In a previous study(1) it was found that, although livers of vit. B₆ deficient rats exhibited markedly reduced glutamate-pyruvate transaminase and cysteine desulhydrase activities, the glutamine- α -keto acid transamination-deamidation reaction proceeded at about equal rates with livers of control and experimental rats.[†] This finding, and our inability to resolve the glutamine enzyme by fractionation, were consistent with the concept that vit. B₆ was not involved in the glutamine- α -keto acid reaction, or alternatively, that affinity of the glutamine system for coenzyme was greater than that of the other enzymes studied. The present study supports the latter interpretation.

Patients given isonicotinic acid hydrazide (INAH) for treatment of tuberculosis devel-

oped a peripheral neuritis, ameliorated by administration of pyridoxine; such patients were also found to excrete in their urine greater than normal quantities of vit. B₆, apparently in a conjugated form involving INAH(2). These findings suggested to us that administration of INAH might represent a useful experimental procedure for depleting an animal of vit. B₆. We have therefore administered large doses of INAH to several groups of rats, and after periods ranging from 8 to 30 days, examined the livers for glutamine transaminase-deamidase activity in the presence and absence of coenzymes.

Methods. Weanling rats of Sprague-Dawley strain weighing 30 to 45 g, were used in Exp. 1 to 4, and adult Buffalo rats weighing 150 to 250 g were employed in Exp. 5 and 6. Animals were housed in individual cages and given water and diet *ad libitum*. *Exp. 1:* 26 rats were given stock Purina Chow diet; 13 of these were given 600 mg of INAH/k/day by gastric tube, and 13 received approximately same amount in drinking water. After

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[†] We have subsequently confirmed this observation using weanling rats of the Sprague-Dawley strain; adult Buffalo rats were used in the earlier work(1).

30 days, 5 animals of each group remained alive. *Exp. 2:* 30 animals received stock diet and 300 mg of INAH/k (by gastric tube) daily for 20 days; all survived. *Exp. 3:* 30 rats given vit. B₆ deficient diet and deoxypyridoxine as previously described(1), as well as 300 mg of INAH/k by tube per day for 14 days; 20 rats survived. *Exp. 4:* 30 rats were given same regimen as in *Exp. 3* for 10 days; 25 rats survived. *Exp. 5:* 39 rats given stock diet and 480 mg of INAH/k by tube per day for 8 days; 26 survived. *Exp. 6:* 60 rats given stock diet and 480 mg of INAH/k by tube for 10 days; 37 rats survived.

Results. Large doses of INAH were employed because preliminary experiments indicated that smaller doses, at least over periods of 10-20 days, did not result in lowered transaminase activity. In general, rats given high doses of INAH were sluggish, developed moist snouts, and failed to gain weight or lost weight (0 to 0.7% body weight/day); death usually occurred shortly after INAH administration and was preceded by convulsions. Typical physical signs of vit. B₆ deficiency did not appear, even in *Exp. 3* and *4*, in which vit. B₆ deficient diet was administered; however, the duration of these studies was relatively short.

Enzyme assays were carried out essentially as described previously(1). The homogenate system consisted of 0.5 ml each of homogenate, L-glutamine (20 μ M), sodium pyruvate (40 μ M), and 0.1 M veronal buffer (pH 7.8), and incubated at 37° for 2 hours. The purified enzymes were isolated and lyophilized(3,4), and assayed in a system consisting of 20 μ M of sodium pyruvate, 10 μ M of L-glutamine, 10 mg of enzyme, in 0.4 ml of 0.025 M veronal buffer; the incubation period was 2 hours. Pyridoxal phosphate and pyridoxamine phosphate were used at a concentration of 100 γ /0.4 ml.

In *Exp. 1*, the livers of the treated rats exhibited greatly reduced glutamine-pyruvate transaminase-deamidase activity and increased glutaminase activity (Table I). Addition of pyridoxal phosphate or pyridoxamine phosphate increased glutamine-pyruvate activity in some instances, but not sig-

TABLE I. Glutaminase and Glutamine-Pyruvate Transaminase-Deamidase Activity of Rat Liver Homogenates.*

Procedure	Substrates†	No. of rats	Range	Mean
Controls	Gl	9	.60-1.52	1.01
	Gl + P	9	2.95-4.54	3.56
INAH by tube	Gl	4	2.16-3.91	2.80
	Gl + P	4	.89-2.90	1.81
INAH in water	Gl	6	1.33-2.49	2.03
	Gl + P	6	.67-2.86	1.91

* Values are expressed in terms of μ M of ammonia formed (corrected for blank ammonia) per ml of homogenate per hr; experimental details are given in the text.

† Gl = Glutamine; P = Pyruvate.

nificantly in others. Because of the variable response to addition of the coenzymes, and because of the possibility that increased glutaminase activity might interfere with the determination of the glutamine-pyruvate reaction, subsequent studies were carried out using purified enzyme preparations obtained from pooled livers of a group of experimental rats and of their untreated controls.

Experiments on several such purified enzyme preparations are described in Table II. These preparations did not possess glutaminase activity, and the effects observed with added coenzymes were readily reproducible. Preparations of the enzyme from livers of INAH treated rats exhibited a considerably reduced activity, and showed a significant increase in activity when pyridoxal phosphate or pyridoxamine phosphate was added. In

TABLE II. Glutamine-Pyruvate Transaminase-Deamidase Activity of Purified Liver Preparations.*

Exp. No.	Procedure†	Initial activity	Activity with coenzymes	
			Pyridoxal PO ₄	Pyridoxamine PO ₄
2	Con.	7.50	8.10	—
	Exp.	3.07	4.50	—
3	Con.	7.44	8.06	8.04
	Exp.	1.49	3.16	3.36
4	Con.	6.09	5.75	5.50
	Exp.	1.38	3.38	3.37
5	Con.	6.46	6.25	—
	Exp.	2.06	5.66	—
6	Con.	5.26	5.73	—
	Exp.	2.44	5.77	—

* See footnote, Table I.

† Con. = Control; Exp. = Experimental.

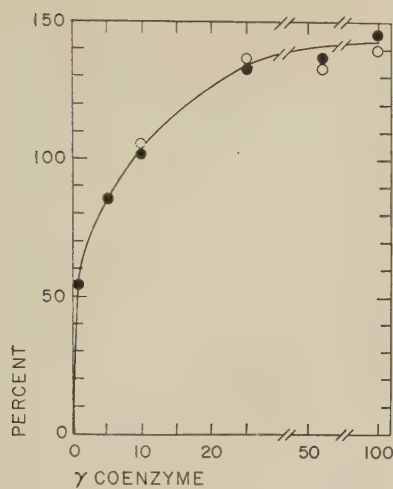


FIG. 1. Effect of pyridoxal and pyridoxamine phosphates on activity. Abscissa: γ coenzyme; ordinate: % increase in activity. Closed circles = pyridoxal phosphate; open circles = pyridoxamine phosphate.

confirmation of earlier studies(1,3,4) values of transaminase activity were in close agreement with those for ammonia formation. Maximal activation was observed with 25 γ of coenzyme (Fig. 1).

Discussion. The present results are consistent with the concept that the glutamine transaminase, like other transaminases, requires a vit. B₆ coenzyme. Failure of earlier attempts to demonstrate this may probably be ascribed to strong binding of the coenzyme by the enzyme. It is apparent that a relatively severe deficiency was required to remove vit. B₆ from this enzyme. It is of interest that concentration of coenzyme required for maximal activation of the partially resolved enzyme is of approximately the same order of magnitude as that required by other transaminases. It thus appears that deamidation of glutamine in this system may be made to depend on the presence of vit. B₆. It is probably the first step of the transamination-deamidation reaction (transamination of glutamine to α -ketoglutaramic acid) which requires vit. B₆. The second step, in which ammonia is formed, is hydrolysis of α -ketoglutaramate to α -ketoglutarate(5).

It has been reported that INAH inhibits *E. coli* arginine decarboxylase(6), tryptophanase(7), and glutamate-oxalacetate trans-

aminase of BCG(8); inhibition was reversed by pyridoxine, which is presumably converted to pyridoxal phosphate in the cell. It was also observed that patients being treated with INAH excreted unusually large amounts of xanthurenic acid in the urine after a test dose of tryptophan(2). The present results appear to provide general confirmation of the concept that this compound has an anti-vit. B₆ effect.[†] It is clear, however, that INAH has other effects (*cf*(9)) some of which may be related to the present observation of increased liver glutaminase and the fact that transaminase activity of preparations obtained from INAH-treated animals was often lower, even with added coenzyme, than that of controls. In 2 experiments in which INAH treatment was of relatively short duration, pyridoxal phosphate restored the activity to virtually control levels. (Exp. 5 and 6, Table II).

Beaton and Ozawa(10) reported that liver extracts of vit. B₆-deficient rats exhibited decreased ability to catalyze the glutamine-pyruvate reaction. Addition of pyridoxal phosphate to the extracts produced only very small and erratic activation.[§] Our present results, which show a clear-cut reproducible *in vitro* effect of pyridoxal phosphate and pyridoxamine phosphate, lead to a conclusion which is not essentially different than that of Beaton and Ozawa, namely that vit. B₆ is involved in the mechanism of glutamine- α -keto acid transamination reaction. The present findings also suggest that INAH treatment may be a useful tool in the study of vit. B₆-catalyzed enzymatic reactions.

[†] We have also found that livers of INAH-treated rats exhibited reduced glutamate-pyruvate and aspartate-ketoglutarate transaminase activities; the control level of activity was restored by addition of pyridoxal phosphate.

[§] It was observed several years ago that livers of very markedly vit. B₆ deficient rats and mice exhibited reduced glutamine transaminase-deamidase activity. It may not be concluded from this evidence that the enzyme specifically required vit. B₆ because addition of pyridoxal phosphate did not increase the activity of enzyme preparations obtained from these very sick animals. (Meister, A., Morris, H. P., and Tice, S. V., unpublished data).

Summary. 1. Liver homogenates prepared from rats given large doses of isonicotinic acid hydrazide (INAH) exhibited reduced glutaminepyruvate transaminase-deamidase activity and increased glutaminase activity. 2. Purified enzyme preparations (free of glutaminase) prepared from livers of INAH-treated rats catalyzed the glutamine-pyruvate reaction at markedly reduced rates compared to controls. Addition of pyridoxal (or pyridoxamine) phosphate produced a considerable increase in activity of the preparations obtained from INAH-treated rats, but not of that of the untreated controls. 3. The evidence supports the concept that vit. B₆ participates in the glutamine transaminase system.

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Effect of Dihydrocholesterol and Soybean Sterols on Cholesterol Metabolism in Rabbit and Rat.* (22167)

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Because considerable evidence exists showing that such sterols as dihydrocholesterol and soybean sterols are absorbed from the gut, it was considered worthwhile to restudy the effects of these sterols on cholesterol metabolism in rabbit and rat. The data in this paper provide an explanation for the conflicting reports heretofore published and demonstrate the fallacy inherent in attempts to produce a negative cholesterol balance by inhibition of cholesterol absorption from the gut.

Experimental. Preparation of sodium acetate-1-C¹⁴. Methylmagnesium bromide was carbonated with C¹⁴O₂. Control diets consisted of Purina Rabbit Chow for rabbits and Purina Laboratory Chow for rats. Sterol diets were prepared by dissolving the requisite

amount of dihydrocholesterol[‡] or mixed soybean sterols[§] in ether, adding the solution to control diets and evaporating the ether. Sera and tissues were prepared for sterol analysis as described previously(1). *Sterol determination.* Digitonin precipitable, Liebermann-Burchard (L.B.) positive sterols were determined by the method of Sperry and Webb(2) and total digitonin precipitable sterols by a modification of the Pollak-Wadler turbidimetric method(3). The latter method was modified as follows: Sterols were precipitated with digitonin in graduated glass-stoppered tube at 10°C. After precipitation the volume was adjusted in the tube, and one drop

[‡] Dihydrocholesterol (Lot no. 105-220 I MRS), generously supplied by Schering Corp., contained 98% Liebermann-Burchard negative sterols.

[§] The mixed soybean sterols (Control no. S-7577 and S-9707), generously supplied by Distillation Products Industries, contained 79% and 94% Liebermann-Burchard positive sterols respectively.

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[†] This work was done during tenure of fellowship as Established Investigator of American Heart Assn.

TABLE I. Recovery of Soybean Sterols from Acetic Acid Solution by Various Methods for Cholesterol Determination.

L.B. positive sterol added, mg		Representative recoveries of L.B. positive sterols							
		Methods							
		Sperry-Webb		Abell <i>et al.</i>		Modified Pollak-Wadler with Acetone-ethanol			
Cholesterol	Soybean sterols	mg	%	mg	%	mg	%	mg	%
.160	—	.170	106	.163	102	.163	102	.169	105
—	.160	.133	83	.150	94	.134	84	.162	101

of polyoxyethylene sorbitan mono-laurate (Tween 20) added to stabilize the suspension. Optical density of the suspension was determined photoelectrically. The digitonide precipitate was removed by centrifugation, and optical density of supernatant determined. Amount of digitonide was computed from the difference in optical densities. For a more complete precipitation of the soybean sterol digitonides it was necessary to change the alcohol in the precipitating medium from ethanol to methanol (Table I). *Feeding experiments.* Male New Zealand white rabbits weighing 2-2.5 kg were used. These were divided into 3 groups (Tables II and III). The first group received control ration. The second received 3 g dihydrocholesterol (DHC) 3 times weekly. The third received 3 g mixed soybean sterols 3 times weekly. Animals were maintained on these diets for 4 weeks at which time they were sacrificed and blood and tissue samples removed. Male rats of Sprague-Dawley strain weighing 200-300 g were divided into 3 groups. The first group received control ration, the second received 1% DHC (Table II), the third received 3% mixed soybean sterols (Fig. 1). Three animals from each group were sacrificed weekly for 5 weeks. Pooled liver samples were removed each time for determination of cholesterol synthesis and sterol content. The sterol contents of control and DHC groups were also determined after 8 weeks on the diet. Rabbits and rats were allowed to eat *ad libitum*, with weight gain determined weekly. There were no significant differences in diet consumption or weight gains between control and experimental animals. *Isotopic experiments.* Liver cell clusters(4) were prepared from livers of control and sterol-fed rabbits and rats; incubation

with C¹⁴-carboxyl-labeled acetate, isolation of cholesterol and analysis of its radioactivity were performed as described previously(5).

Results. The use of proper analytical procedure for sterol analysis was essential in this study. Soybean sterols, which consist largely of isomers of sitosterol and some stigmaterol, form a digitonide and give the L.B. reaction (6). We found that recovery of soybean sterols from solution by the commonly employed technics of Sperry and Webb(2) and Abell *et al.*(7) was incomplete. Table I shows the efficiency of recovery of soybean sterols by these methods as well as by the modified Pollak-Wadler procedure. The recovery of soybean sterols by the Sperry-Webb method and modified Pollak-Wadler procedure with acetone-alcohol was poor. The reason for the failure of recovery was solubility of soybean sterol-digitonin complex in 1:1 acetone-ethanol. In gravimetric experiments we have found that washing 28.4 mg of soy sterol digitonides with two 5-ml aliquots of 1:1 acetone-ethanol dissolved away 61% of the digitonides.

Recovery of soy sterols by the method of Abell *et al.* was better. However, the determinations in Table I were performed with a minimum exposure to the stream of air used in the method to evaporate petroleum ether extract to dryness. We have determined, in a manner similar to that employed by Sperry and Brand(8), that soybean sterols were 2 times as sensitive to air oxidation as cholesterol; like cholesterol, air oxidation destroyed soy sterols' ability to give the L.B. reaction. It is probable then that loss of soybean sterols during analysis by the method of Abell *et al.* resulted from air oxidation and

TABLE II. Liver and Serum Sterols of Rabbits Fed Dihydrocholesterol (DHC) and Incorporation of Sodium Acetate-1-C¹⁴ into Cholesterol by Excised Livers* of Rabbits and Rats Fed DHC.

Group	Animal	Diet	Duration of feeding, wk	Serum, mg %			Liver, mg/g			Specific activity of liver cholesterol [§]
				A	B	C	A	B	C	
				Total digi- tonin pre- cipitable sterols	Cholesterol†	DHC‡	Total digi- tonin pre- cipitable sterols	Cholesterol†	DHC‡	
I	Rabbit 1	Control	4	42	50	—	5.3	4.6	.7	—
			4	33	36	—	5.6	5.1	.5	306
II	3	9 g DHC/wk	4	100	72	28	21.3	12.7	8.6	34
			4	87	59	28	18.6	10.5	8.1	—
I	4 rats	Control	8	—	49	—	5.8	5.6	.2	387
II	4 "	1% DHC	8	—	30	—	8.4	5.2	3.2	127

* Each vessel contained 3-4 g (wet wt) of liver cell clusters, 40 ml of buffer and 5 mg of CH₃C¹⁴OONa • 3H₂O (5.1 mc/mM),

† L.B. positive sterol as determined by method of Sperry and Webb.

‡ Digitonin precipitable, L.B. negative sterol (column A minus column B).

§ Counts/min./mg of cholesterol/g dried liver.

|| Determined gravimetrically as the digitonide.

that this loss may be far greater than shown in Table I. It should be added, however, that substitution of N₂ for air in the petroleum ether evaporation step in the Abell *et al.* procedure obviates this difficulty. From this evidence it seems clear that neither the Sperry-Webb nor Abell *et al.* procedures for cholesterol determination is adequate for analysis of soybean sterols. One may further conclude that use of these methods to determine serum and tissue total L.B. positive sterols may result in falsely low results when soybean sterols are present. For this reason reports, based on these methods, of a lowering of cholesterol levels after feeding soybean sterols may be suspect if soybean sterols are present in the body.

As the last column of Table I demonstrates, substitution of methanol in Pollak-Wadler type procedure provided the best recovery of soy sterols from solution.

Table II gives results of feeding experiments with DHC. It can be seen that DHC, which forms a digitonide but is L.B. negative (9), was found in sera and livers of rabbits and in livers of rats, thus indicating its absorption. Under these feeding conditions, that portion of the digitonin precipitable sterol which was L.B. negative was considered to be DHC. This assumption was strengthened by the fact that increased saturated sterol fractions(10) from the livers of rabbits 3 and 4 had infrared absorption spectra characteristic for DHC. It is of interest to note that the liver cholesterol content of rabbits 3 and 4 was elevated as well as the DHC content. This may have resulted from ability of liver to convert DHC to cholesterol(11). The last column of Table II demonstrates that livers of DHC-fed rabbits and rats have a decreased ability to incorporate labeled acetate into cholesterol. These results confirm the findings of Siperstein(12), Gould *et al.*(13), and Ivy *et al.*(14) that DHC is absorbed by the rat and further demonstrate absorption in the rabbit. In agreement with the report of Gould *et al.*(13) an inhibition of hepatic cholesterol synthesis in rats fed DHC has been demonstrated and a similar inhibition shown in the rabbit. The greater degree of inhibi-

TABLE III. Liver and Serum Sterols of Rabbits Fed Soybean Sterols for 4 Weeks and Incorporation of Sodium Acetate-1-C¹⁴ into Cholesterol by Their Excised Livers.*

Group	Animal	Diet	Serum, mg %			Liver, mg/g			Specific activity of liver cholesterol [§]
			Total digitonin precipitable sterols (methanol)	Cholesterol [†]	Soybean sterols [‡]	Total digitonin precipitable sterols (methanol)	Cholesterol [†]	Soybean sterols [‡]	
I	1	Control	26	25	—	5.7	5.6	—	607
	2		25	25	—	6.8	6.0	—	457
III	1	9 g soybean sterols/wk	40	45	0	10.2	5.1	4.6	155
	2		47	49	0	9.3	5.5	3.3	—
	3		32	35	0	10.0	6.3	3.2	242
	4		26	26	0	7.1	6.2	0.4	545

* Each vessel contained 3-4 g. (wet wt) of liver cell clusters, 40 ml of buffer and 5 mg of CH₃C¹⁴. OONa · 3H₂O (5.1 mc/mM).

† L.B. positive sterol as determined by method of Sperry and Webb.

‡ Digitonin precipitable (in methanol), L.B. positive sterol, corrected for cholesterol and for L.B. negative sterol content.

§ Counts/min./mg of cholesterol/g dried liver.

tion of cholesterol synthesis demonstrated in our animals undoubtedly reflects the longer feeding period for the sterol. Although atherosclerotic lesions of the aorta were not noted in our rabbits after a 4-week feeding

period, it has recently been reported that a 0.5% DHC diet, fed to cockerels for 6 months, produces aortic atherosclerosis(15).

Table III gives results of rabbit feeding experiments with soy sterols. It can be seen that the total liver sterols of rabbits 1, 2, and 3 of Group III are increased. As determined here, that fraction of total digitonin precipitable sterol (in methanol) which was neither cholesterol nor saturated sterol was considered to be soybean sterols. The validity of this assumption was demonstrated by the fact that infrared absorption spectra of the 1:1 acetone-ethanol soluble fraction of digitonides from livers of Group III rabbits 1, 2, and 3 were identical with infrared absorption spectrum of original mixed soybean sterols. It is of interest that rabbits fed soy sterols did not have measurable levels of these sterols in their serum. The last column of Table III shows that in livers of soy sterol diet rabbits 1 and 3 there was inhibition of cholesterol synthesis from C¹⁴-labeled acetate. On the other hand Group III rabbit 4, which absorbed little soy sterol, showed a normal rate of cholesterol synthesis.

All rabbits receiving 9 g soy sterols per week for 4 weeks had atheromatous lesions of the aorta at autopsy. Infrared absorption spectra of the acetone-ethanol soluble fraction of the digitonides obtained from aortas of the rabbits in Group III also were identical with the spectrum of the dietary soy sterols. It is

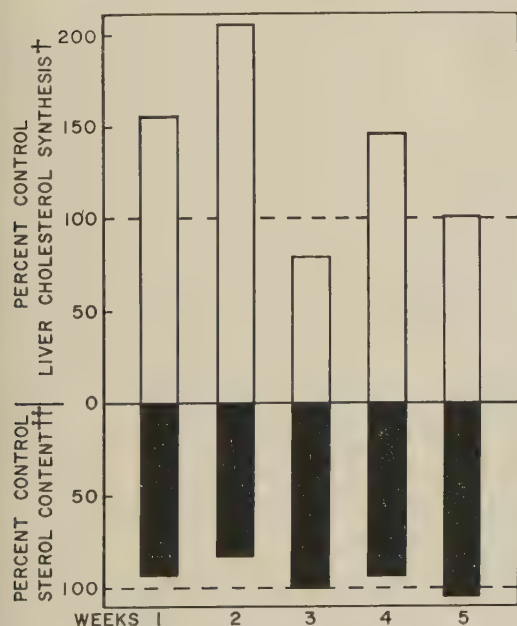


FIG. 1. Hepatic cholesterol synthesis and liver sterol content of rats fed a 3% soy sterols diet.

$$\begin{aligned}
 & \frac{\text{C.P.M./mg cholesterol/g soy liver}}{\text{C.P.M./mg cholesterol/g control liver}} \times 100 \\
 & \frac{\text{Total digitonin precipitable sterols/g soy liver}}{\text{Total digitonin precipitable sterols/g control liver}} \times 100
 \end{aligned}$$

thus apparent that soy sterols are both absorbed and atherogenic.

Fig. 1 shows the effect of a 3% soy sterols diet on the liver sterol content and rate of hepatic cholesterol synthesis in the rat. It can be seen that moderate reduction in liver cholesterol levels produced in the first 2 weeks of diet was associated with a compensatory increase in hepatic cholesterol synthesis. This increased synthesis was sufficient to restore and maintain at normal the liver cholesterol levels for the next 3 weeks of the experiment. These findings are identical with results obtained when rats were fed large amounts of a cholesterol metabolizing *Escherichia coli* which destroyed biliary cholesterol passed into the gut(16). Negative cholesterol balance, produced by either steroid or non-steroid interference with cholesterol reabsorption, is counteracted by an increase in endogenous cholesterol synthesis.

None of the livers from rats fed a 3% soy sterol diet contained a measurable amount of soy sterols. There would thus seem to have been little absorption of these sterols. The transitory reduction in liver cholesterol seen in the first 2 weeks of the experiment undoubtedly resulted from competitive inhibition of cholesterol absorption by the non-absorbable soy sterols(17).

These data demonstrate absorption of soy sterols in rabbits. It has been shown that in rabbits soybean sterols produce a depression of cholesterol synthesis resembling that seen after feeding cholesterol(18), dihydrocholesterol, and other related sterols(12).

Discussion. It is possible to conclude from our data that there is no reason to expect a reduction of tissue cholesterol levels in man by the use of dihydrocholesterol and soybean sterols. The rabbit experiments further suggest that ingestion of such substances may be hazardous.

Summary. 1. Dietary dihydrocholesterol and soybean sterols were absorbed by rabbits, and soy sterols produced atherosclerosis in these animals. 2. Livers of rabbits and rats

fed dihydrocholesterol and of rabbits fed soybean sterols had an impaired ability to incorporate C^{14} -labeled-acetate into cholesterol. 3. Rats fed a 3% soybean sterols diet did not absorb significant amounts of these sterols. Soy sterols failed to produce a lowering of total liver sterol levels in rats.

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Inhibitory Effect of Protamine on Action of Intravenous Thromboplastin in Dogs.* (22168)

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(Introduced by M. Wolman.)

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Slow intravenous infusion of thromboplastin in dogs and other animals produced intravascular clotting resulting in deposition of fibrin on walls of blood vessels(1-7). With prolonged infusion, fibrinogen disappears from blood, while other clotting factors such as prothrombin and proaccelerin (plasma-Ac-globulin, labile factor) are only partially reduced(4,6). Animals recover and regain a normal fibrinogen level in 2-6 days(5-7). Rapid intravenous injection of thromboplastin induces formation of large thrombi near site of injection and causes prompt death of the animals(1,5,6). Attempts have been made with varying success to counteract the effects of thromboplastin *in vivo* with clotting inhibitors such as heparin(1,2), Congo Red, serum(2), soy bean trypsin inhibitor(3), alpha tocopherol phosphate and inositol phosphate(8), and with thromboplastin splitting enzyme, thromboplastinase(9). Natural basic polypeptide protamine and some synthetic basic poly-amino acids were found recently to have antithromboplastic activity (10-12). They also accelerate clotting of fibrinogen by thrombin(12-14). However, when added to whole blood, coagulation is retarded as their antithromboplastic activity predominates.

The present communication deals with the influence of protamine on defibrinating and lethal effects of intravenous thromboplastin.

Materials and methods. *Thromboplastin.* This term refers to an extract in 0.9% NaCl of homologous, acetone dehydrated brain prepared according to Ratnoff and Conley(6). The fresh extract was diluted to give clotting time of 13-14 seconds in one stage prothrombin assay of Quick using plasma of dog to be infused. Protamine was obtained from Nutritional Biochemicals Corp. Solutions of 5

mg/ml were made in 0.9% NaCl, pH 7.4. Healthy mongrel dogs of either sex weighing 5 to 17 kg were used. Sodium pentobarbital (3% solution in 0.9% NaCl), was injected intraperitoneally, 1 ml/kg. Anesthesia was maintained during experiments by further doses as required. *Administration of thromboplastin and protamine.* Surgical sterility was not maintained. Femoral artery and vein were exposed on both sides. In slow infusion thromboplastin was introduced into femoral vein from calibrated burette. The first 50 ml were infused at 2-3 ml/min and thereafter at 4-5 ml/min. Rapid injection of thromboplastin was given at approximately 1 ml/sec. At end of experiments, incision sites were sutured and 500000 units penicillin and 0.5 g streptomycin administered intramuscularly daily for 3 consecutive days. Protamine, when given simultaneously with thromboplastin, was infused into opposite exposed femoral vein at 1 ml/min. When protamine was given prior to infusion of thromboplastin it was injected at the rate of 2-3 ml/min. Whole blood clotting time. Blood samples were taken from femoral artery. Two aliquots of 1 ml were put in glass tubes at 37°C. Clotting time of blood in the second tube was recorded. Thrombin clotting time was employed as a quick indication of presence of fibrinogen. One ml of blood was added to glass tube containing 5 units of Thrombin (Upjohn) in 0.1 ml 0.9% NaCl. After careful mixing the tube was put into water bath at 37°C. Blood was considered depleted of fibrinogen if no clot formed during 3 minutes. *Determination of fibrinogen.* One ml blood was collected into solid oxalates(15) and plasma separated by centrifugation. The method of Ware *et al.*(16) was adapted for analysis of fibrinogen in 0.1 ml of plasma.

Results. *Inhibition by protamine of defibrinating effect of thromboplastin.* Eight

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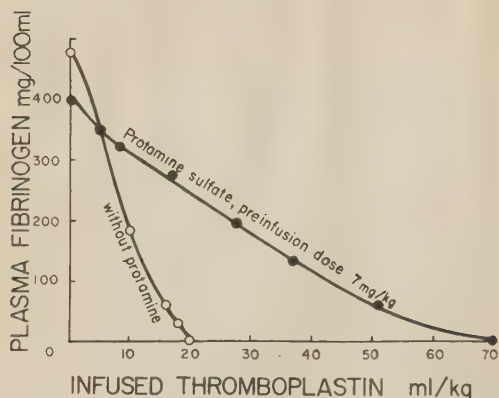


FIG. 1. Representative experiment showing inhibitory effect of protamine administered intravenously prior to infusion of thromboplastin on the decrease of plasma fibrinogen. Mean rate of thromboplastin infusion was 3.5 ml/min. An 8 kg dog was used.

normal dogs were given a slow infusion of thromboplastin, which resulted in a gradual decrease of their plasma fibrinogen level. Whole blood clotting times were elevated to 20-30 min soon after beginning of infusion as compared with 6-8 min in preinfusion samples. When the quantity of infused thromboplastin was near 20 ml/kg, blood became incoagulable. In some dogs a sudden drop in clotting time occurred in samples taken just before total defibrination, presumably due to presence of thrombin in circulation. Some of these samples clotted although they were mixed immediately with oxalate.

Ten dogs received protamine and were divided into 2 groups. The first group of 5 dogs was given 7-10 mg/kg protamine by slow injection; no untoward reactions or changes in plasma fibrinogen level or blood clotting time were observed with amounts and rates of injection employed. Thromboplastin infusion was started 5 min. after injection of protamine. Decrease in plasma fibrinogen level was markedly slowed (Fig. 1) and total defibrination required more than 3 times as much thromboplastin per kg as compared with dogs which did not receive protamine. Blood clotting times were similar to control dogs. Fibrinolysis could not be detected in spontaneously clotted blood at various intervals during thromboplastin infusion into protamine treated and control animals. Nor was

lytic activity observed on addition of fibrinogen free blood, obtained at the end of infusion, to a clotted preinfusion sample of the same dog.

In the second group of 5 dogs the fibrinogen level was lowered to nearly half normal value by slow infusion of thromboplastin. Protamine was then infused. Fig. 2 shows, that plasma fibrinogen remained fairly constant during simultaneous infusion of both thromboplastin and protamine. There was no significant decrease in fibrinogen level even after infusion of protamine was terminated and infusion of thromboplastin was continued to a total of 70 ml/kg.

Effect of repeated infusions of thromboplastin and protamine. All defibrinated dogs recovered from the effects of slow infusion of thromboplastin (20 ml/kg) and their plasma fibrinogen returned to normal within a week. Several of these dogs were twice reinfused with thromboplastin at monthly intervals and approximately the same doses were needed for repeated defibrinations.

In another group of previously defibrinated dogs, protamine followed by thromboplastin was given one month later. The amount of thromboplastin (60 ml/kg), required for the second defibrination, was similar to that needed to produce first defibrination of protamine pretreated dogs (Fig. 1). After an-

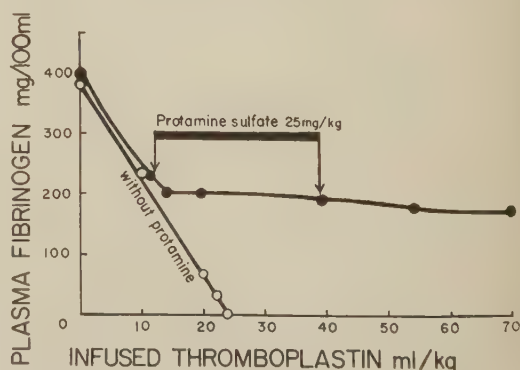


FIG. 2. Representative experiment showing arrest of plasma fibrinogen decrease resulting from administration of protamine to thromboplastin infused dog. Protamine infusion was started when 12 ml/kg thromboplastin had been given and terminated when the quantity of simultaneously infused thromboplastin reached 39 ml/kg. Mean rate of thromboplastin infusion was 4 ml/min. A 10 kg dog was used.

other month when only thromboplastin was infused, the usual dose of 20 ml/kg was again sufficient to render them fibrinogen free. These experiments indicate that repeated infusions did not cause development of either refractoriness or hypersensitivity to thromboplastin and that treatment with protamine did not produce any lasting resistance to the defibrinating action of thromboplastin.

Protecting action of protamine against lethal effect of rapid injection of thromboplastin. Rapid injection of thromboplastin of 5 ml/kg caused almost immediate apnea and death in 10 out of 14 dogs; 2 dogs died during the next 4 hours without recovering from anesthesia, while 2 more survived the injection. An illustrative experiment is described below: Respiration rate of 7 kg female dog under pentobarbital anesthesia was 18/min and pulse 120/min, clotting time 8 min and plasma fibrinogen 380 mg/100 ml. 35 ml of thromboplastin were injected into exposed femoral vein in 40 seconds. At end of injection breathing was rapid and shallow (60/min) then became sporadic and laboured. 75 seconds later breathing ceased and pulse rate was 15/min. At this time, a blood sample showed: clotting time 10 min, fibrinogen 285 mg/100 ml. 135 sec. after injection the heart stopped beating. Pathological examination revealed a blood clot in femoral vein, inferior vena cava, right atrium and ventricle and pulmonary artery, with marked dilatation of right heart. Protamine in doses of 7-10 mg/kg was slowly injected into 14 dogs. Five minutes later the dogs received a rapid injection of thromboplastin as described above. Soon afterwards some showed transient dyspnea, but none died. There was negligible decrease in plasma fibrinogen and recovery of dogs seemed complete. In one of 2 dogs sacrificed 10 days after injection, fibrin clots were found on wall of the right atrium. *Comment.* The present results show that protamine has the ability to counteract the effects of intravenous thromboplastin in dogs. It increases the amount of thromboplastin required for defibrination when injected prior to thromboplastin; it arrests the decrease of fibrinogen when adminis-

tered simultaneously with thromboplastin and it protects animals against lethal effect of rapid injection of thromboplastin.

Inactivation of thromboplastin by protamine or by synthetic poly-arginine(17) *in vitro* is associated with the formation of a precipitate. This results from a salt-like interaction between basic guanidine residues of numerous arginine groups(18) of protamine and the acidic phospholipid components of thromboplastic protein(19). It may be assumed that a similar interaction of protamine with thromboplastin takes place *in vivo*.

Inhibitory effect of protamine infusion on intravenously administered thromboplastin suggests its application in clotting disorders which involve entry of thromboplastic material into the blood stream. Such a mechanism has been supposed to be the cause of afibrinogenemia in abruptio placentae(1,20), in amniotic fluid embolism(21,22) and in some cases of intrauterine death(23,24).

Summary. 1. Intravenous administration of protamine markedly increases the amount of slowly infused thromboplastin required for defibrination of dogs. Protamine given during infusion of thromboplastin, to a dog already partially depleted of fibrinogen, arrests further decrease in plasma fibrinogen level. 2. Intravenous administration of protamine protects dogs from the lethal effect of subsequent rapid injection of thromboplastin. 3. Mode of action of protamine and possible clinical utilization of its antithromboplastic properties are discussed.

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Variation in Reduced Glutathione in Bloods of Inbred Lines of Chickens.* (22169)

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There can be no doubt that the reduced glutathione (GSH) levels of blood and other tissues are influenced by the adrenal, thyroid, pituitary, pancreatic, and parathyroid hormones(1). There is a paucity of data, however, which evaluate the extent that activities of endocrine glands are under hereditary control and, therefore, the extent that variations in genetically controlled activities of endocrine glands regulate chemical activities of the cells.

Proof of genetic control of vital biochemical reactions in living organisms has been demonstrated principally by results of studies conducted on monoploid microorganisms(2,3). In higher animals, such evidence has resulted from studies of physiological abnormalities, primarily in the human being, from studies of pigment development and body coloration, or the presence or absence of a chemical con-

stituent such as atropinase or a β -globulin in the sera(4-8). Williams *et al.*(9) have suggested a quantitative individuality in metabolism of humans and rats on the basis of variation in patterns of excretion of metabolites in urine. Breed differences in plasma or serum alkaline phosphatase in chickens(10), in thyroxine secretion rate of chickens(11,12), and in total body glutathione concentration in 2- to 14-day-old chicks(13) lay a broad basis for the study of biochemical genetics in chickens, but the majority of such studies has not dealt with these biochemical factors as bases for biological individuality.

Genetically controlled individual quantitative differences in biochemical constituents should tend to become fixed by inbreeding and, as a result of this increase in intra-line genetic uniformity, differences between inbred lines become more easily demonstrable. With availability of inbred lines of chickens within a single breed (White Leghorn) in our

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laboratories, studies have been initiated on constancy of and genetic control of the concentration of GSH in mature chickens.

Methods and materials. Blood levels of reduced glutathione have been determined for mature male and female birds of 3 inbred lines, *viz.*, Lines 22, 23, and 24 of White Leghorns, maintained by the Poultry Husbandry Department, A. and M. College of Texas. These birds were hatched during 1952, 1953, and 1954 seasons. All these lines were inbred to about the same extent. The average inbreeding coefficient of birds used in these experiments was 0.53. Whole blood GSH was estimated by the method of Grunert and Phillips(14). The method was modified by increasing the volume of hemolyzing saponin solution to 4 ml for each ml of blood. Using potassium oxalate as anticoagulant, blood samples were collected by venepuncture and were placed immediately into ice bath at about 0°C. Duplicate analyses were made on samples within 8 hours after collection. Hemoglobin values were determined colorimetrically as oxyhemoglobin and red cell volumes were estimated after centrifugation of blood samples for 45 minutes at 1400 x g in Wintrobe tubes.

The data were statistically analyzed according to the methods outlined by Snedecor(15) to evaluate the significance of observed differences. Repeatability estimates were calculated as product-moment correlation coefficients between results of successive determinations. Coefficients of variation were calculated as standard deviation divided by the mean and reported as percentages.

Results. Estimates of repeatability. One group of 18 males of Line 22, housed on the range, had a mean GSH level of 48.8 ± 1.8 mg/100 ml blood. Analyses made on the same birds 5 weeks later showed a mean value of 51.8 ± 1.8 mg/100 ml. The correlation or estimates of repeatability between these successive determinations on the same birds was found to be 0.58 and is statistically significant ($p < 0.05$). The coefficient of variation for the first set of determinations was 15.8% and 14.4% for the second. In a second group of males from the same line, the

TABLE I. Repeatability Estimates of Reduced Glutathione Levels in Blood of Lines 22, 23, 24, and 22 x 24 Females. Ten birds in each series.

Line	Date	Avg glutathione, mg/100 ml	Estimate of repeatability*	Coeff. of variation, %
22	3/17/55	$33.4 \pm 1.6^\dagger$		14.82
	26	39.5 ± 2.1	.90	16.63
23	17	21.7 ± 1.0		15.18
	26	25.7 ± 1.1	.78	13.90
24	17	26.9 ± 1.6		18.83
	26	33.0 ± 1.8	.88	16.89
22 x 24	18	29.0 ± 1.1		11.79
	29	34.9 ± 1.9	.79	16.88

* Significant at .01 level of probability.

† Stand. error of the mean.

mean GSH levels observed from subsequent determinations on 40 birds were 50.9 ± 1.1 and 63.0 ± 1.4 mg/100 ml blood, respectively. Coefficient of variation was 13.6% in the first, and 14.4% in the second determination. Again the estimate of repeatability between these determinations was found to be 0.58, a correlation which was highly significant statistically ($p < 0.01$). Just prior to the second determination, the 40 males described above were moved from one set of open-air quarters to another. In view of the fact that no dietary change was made, it might be possible that the increased level of GSH observed in the second determination could be due to environmental changes. Regardless of whether the difference between means was the result of environmental effects or a possible uncontrolled analytical error, the significant correlation between determinations was indicative of an individuality in the level of blood GSH within an inbred line.

Subsequent GSH determinations made on 10 mature females from each of inbred White Leghorn Lines 22, 23, and 24 were used to calculate estimates of repeatability of GSH measurement in the inbred female. In addition, successive determinations were made on 10 females produced from a cross of Line 22 males on Line 24 females. All of these birds were individually caged. Repeatability estimates for all these lines were highly significant (Table I) and ranged from 0.78 to 0.90. Again an increase of 18 to 23% in the aver-

TABLE II. Average Blood Levels of Glutathione of Mature Males from Inbred White Leghorn Lines 22, 23, and 24.

Line	Avg glutathione, mg/100 ml blood				No. of birds
	1952	1953	1954	3 yr avg	
22	51.7 $\pm$ 1.1	54.4 $\pm$.9	51.5 $\pm$ 2.8	53.3 $\pm$.7	85
23*	44.4 $\pm$ 1.6	41.2 $\pm$ 1.9	43.3 $\pm$ 2.0	43.0 $\pm$ 1.1	18
24	56.7 $\pm$ 1.1	50.3 $\pm$ 1.3	53.8 $\pm$ 2.0	52.2 $\pm$ 1.1	59

* Highly significantly lower ($p < 0.01$) than other two lines.

age GSH level in the second determination was noted. When the second GSH analyses were made, the environmental temperature was much lower than temperatures existing during first experimental period and possibly may account for the differences. While environmental effects could have affected the level of GSH, the rank order of levels in females as with the results with males, described above, was not appreciably altered.

Genetic Control. Levels of GSH were determined in 162 mature males of the 3 inbred White Leghorn Lines 22, 23, and 24. These birds ranged from about 10 to approximately 30 months of age when the determinations were made. The average levels, expressed in mg of glutathione/100 ml blood, are presented in Table II. These data show that average GSH levels of males from Line 23 were consistently lower over the 3-year period than levels of Lines 22 and 24. The hereditary control of the blood GSH level thus becomes apparent. The data were statistically analyzed for effects of age, inbred line, and dams and sires. Contrary to findings with cattle (16, 17), age was not a significant factor in determining the level of GSH in blood of these birds. The analysis did demonstrate, however, that the lower levels of GSH in birds of Line 23 were highly meaningful statistically ($p < 0.01$).

The average GSH levels determined for 18 females from each inbred Line 22, 23, and 24, and 22 females from Line 22 x 24 crosses were 32.0 ± 1.2 , 22.5 ± 0.8 , 27.3 ± 1.3 , and 28.7 ± 0.9 mg/100 ml blood, respectively. The differences between the 3 inbred lines were highly significant statistically. The significant line differences with both males and females provide strong evidence for the genetic control of quantitative differences in blood GSH. It is to be noted also that the

levels in females were considerably lower than in males. The glutathione level observed for Line 22 x 24 females fell between the levels of parental lines. Thus, there was no evidence of a heterotic effect on GSH level. Unfortunately, representatives from other crosses between these lines were not available.

Effect of Erythrocyte Concentration. The erythrocyte is the primary source of GSH found in blood, and therefore, variation in number of red cells could account for part of the differences observed in GSH levels. In 52 males from Line 22 with a mean GSH level of 51.2 ± 0.9 mg %, the average hemoglobin content was 12.14 ± 0.23 g/100 ml blood. The correlation of 0.62 between concentrations of these 2 substances was highly significant statistically ($p < 0.01$). Yet, in spite of the fact that the erythrocyte is the primary source of GSH in blood, differences in red cell number (indicated by hemoglobin content) accounted for only about 38% of the variation observed in the level of GSH of these males.

GSH levels, hemoglobin content, and red cell volumes were determined for a group of males and females from each inbred line. It is to be noted from the data (Table III) that GSH content of female bloods averaged from 26 to 34% lower than bloods from males of the same lines. However, the hemoglobin content was from 33 to 38% lower and the red cell volume was 32 to 37% lower in females. The major portion of the sex difference in GSH levels within a line was primarily the result of variation in number of erythrocytes as indicated by relative levels of hemoglobin and red cell volume. The decrease in total GSH due to reduction in volume of red cells in the female is partially offset by a 5 to 10% increase in GSH per volume of red cells. The average hemoglobin values and

TABLE III. Average Glutathione Levels, Hemoglobin Values, and Red Cell Volumes of Mature Males and Females from Inbred Lines 22, 23, and 24.

Line	Sex	Glutathione, mg/100 ml	Hemoglobin, g/100 ml	Red cell vol, %	Glutathione- hemoglobin ratio, mg/g	Glutathione conc. in red cells, mg/ml
22	♂	53.5 ± 1.6 (9)*	12.6 ± .5	41.9 ± 1.0	4.25	1.28
	♀	39.5 ± 2.1 (10)	8.4 ± .3	28.5 ± 1.0	4.70	1.39
23	♂	37.9 ± 1.0 (10)	12.3 ± .3	41.1 ± 1.1	3.08	.92
	♀	25.2 ± 1.5 (7)	7.9 ± .4	26.0 ± 1.1	3.19	.97
24	♂	48.8 ± 2.1 (9)	12.7 ± .4	42.3 ± 1.1	3.85	1.15
	♀	34.8 ± 1.1 (8)	7.9 ± .2	27.3 ± .8	4.42	1.27

* The number enclosed in parenthesis indicates the number of birds with all 3 values.

red cell volumes of the 3 inbred lines within sexes are essentially the same. Therefore, the observed line differences in GSH levels are clearly due primarily to different concentrations of GSH within red cells. It may be concluded that while environment and experimental treatment could have some effect, concentration of reduced glutathione within the erythrocyte is conditioned to a large extent by heredity.

Summary. 1. Significant correlations were obtained between successive reduced glutathione (GSH) determinations on the same birds. This was true in both males and females and indicated a high degree of constancy for the GSH level in individual chickens. 2. The blood level of GSH was determined in 162 mature males of 3 age groups from 3 inbred lines of White Leghorns. Age was not a significant factor, but one line had a highly significantly lower GSH level than the other 2 lines. Differences between the GSH levels in females from all 3 inbred lines were highly significant statistically. 3. Females of the 3 inbred lines had much lower GSH levels than the males. However, hemoglobin content and red cell volume of females were also much lower. Since the source of GSH is almost wholly the red cells, the difference in GSH level between males and females within a line could be attributed to relative numbers of red cells present in the sexes. Line differences in males and females appear to result from different concentrations of GSH within the red cell. The data provide evidence that the GSH concentration within the erythrocytes of mature chickens is

to a very large extent genetically controlled.

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Cold-Adapted Animals. I. Changes in Blood Clotting and Electrophoretic Properties of Rabbit Plasma.* (22170)

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Changes in the clotting mechanism as a result of prolonged exposure to low temperatures, "cold adaption," have occasionally been noted(1) for non-hibernating mammals and people. More marked changes have been observed during hibernation or aestivation in mammals such as ground squirrel or hamster (1-4). During preliminary studies on cold adaption in rabbits, it was observed that plasma clotting times were prolonged, prothrombin times decreased, platelet material increased, and fibrin yield was greater. The present studies were undertaken to extend these experiments. Some changes in technic were made and earlier results, in qualitative agreement with the present ones are not included in the Tables. Electrophoretic studies, initially made to determine if abnormalities occurred in the fibrinogen component, have been included.

Methods. Conditioning of rabbits. Six young healthy male rabbits, about 3 kg, were placed in a cold room at 4°C for 2 months. During the first month animals were progressively clipped until hair remained only on the extremities. Animals were maintained in this condition by regular clippings for the last month. Animals gained weight at a slightly higher rate than controls and appeared healthy throughout the experiment. A similar number of controls were kept, unclipped, in the usual animal quarters. **Hematological technics.** 1. *Bleeding.* All glassware and needles were freshly siliconed before use (Desicote, Beckman Instrument Co.). Three ml of blood were drawn by cardiac puncture and discarded. A 50-ml syringe was placed on the needle, temporarily left *in situ*, and 40 ml blood was rapidly withdrawn. Thirteen ml of this blood was immediately transferred to

paraffined 15-ml conical centrifuge tube, the remaining 27 ml transferred to 50-ml centrifuge tube containing 3 ml of 0.1 M sodium oxalate, and stirred by gentle inversion, using Parafilm as cover. 2. *Blood clotting times.* The 15-ml centrifuge tubes stood at room temperature for 10 minutes, and then tilted every 30 seconds until no flow occurred on complete inversion. 3. *Erythrocyte and platelet counts.* Portions of oxalated blood were diluted in Rees-Ecker solution and counted in a Neubauer counting chamber. 4. *Plasma clotting times.* The oxalated blood was spun for 5 minutes at 1500 rpm (133 g) and a portion of platelet-containing plasma withdrawn (samples "A" in Table II). Blood was then spun for 45 minutes more at 3000 rpm (1600 g) and platelet-poor plasma removed, (samples "B" in Table II). A portion of each sample was diluted with equal volume of 1% saline, and 0.1 ml transferred to 13 x 100 mm test tube and incubated for 150 seconds at 37°C. An equal volume of 0.0139 M CaCl₂† at 37°C was then added and the tube sat for

TABLE I. Platelet and Erythrocyte Counts, and Hematocrits of Normal and Cold-Adapted Rabbits.

Rabbit No.	Platelet counts (× 1000)	Erythrocyte counts (× 1000)	Hematocrits, %
Normal rabbits			
993	322	5170	43.2
994	317	5930	44.5
995	417	5830	41.0
996	383	5960	41.6
997	389	4960	39.2
Mean	366	5570	41.9
Cold-adapted rabbits			
976	395	5790	41.0
977	382	6730	47.4
978	485	6340	45.0
979	399	5210	43.9
980	487	5720	47.4
982	348	5620	45.5
Mean	416	5900	45.0
Mean % change in cold	+13.7	+5.9	+7.4

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TABLE II. Clotting Data on Blood from Normal and Cold-Adapted Rabbits.

Rabbit No.	Blood clotting time (min.)	Plasma clotting time (sec)		Prothrombin time (sec)	Fibrin yield (g/100 ml)
		A*	B*		
Normal rabbits					
993	26.5	87	173	66.0	.44
994	29.5	87	170	58.5	.52
995	24.5	77	150	47.0	.44
996	10.0	67	160	—	.43
997	25.0	77	400	57.5	.37
Mean	23.1	79.0	210.6	57.3	.44
Cold-adapted rabbits					
976	29.0	87	273	—	.47
977	24.0	77	173	36	.59
978	21.5	117	733	38	.59
979	32.0	77	143	39	.60
980	38.0	93	263	32	.81
982	50.0	67	113	40	.66
Mean	32.4	86.3	283.0	37.0	.62
Mean % change in cold	+40.2	+9.3	+34.3	-35.4	+41.0

* Samples "A" spun at 1500 rpm (133 g) for 5 min.
 "B" " " 3000 " (1600 g) " 45 "

50 seconds. It was tipped thereafter every 10 seconds, and the time for a firm clot to appear was taken as the end point. 5. *Fibrin determinations.* Clots from plasma clotting time determinations sat at room temperature for 16 hours, and then shaken free from walls of tubes, and the tubes filled with 1% saline. They were spun at 3000 rpm (1600 g) for 45 minutes and fluid above the collapsed clot drawn off through a capillary by suction. The fibrin mat was washed in this fashion 3 more times, and then analyzed for nitrogen by Micro-Nessler technic of Lanni(5). Conversion to protein was made by using the 6.25 factor. 6. *Prothrombin time.* These values were obtained in triplicate according to the one-stage method of Campbell, Smith, Roberts, and Link, as adapted by Holburn(6). Dilutions of platelet-free plasma were 5.0, 8.34, 12.5 and 20%, and values presented were taken from the log log plots of clotting time vs plasma concentration at the 10% plasma point. 7. *Total protein determinations* were done by the Micro-Nessler method of Lanni(5) on triplicate 0.2 samples of 1:100 dilution of plasma. 8. *Electrophoretic anal-*

yses were made with a Perkin-Elmer apparatus using pH 8.6 Veronal buffer, ionic strength 0.1.

Results. From the data presented in Table I it appears that cold-adapted animals develop an increase in platelet[†] and erythrocyte counts and in hematocrit.

Table II presents results of clotting studies. It can be seen that in cold adapted rabbits blood and plasma clotting times are increased, while prothrombin time decreased. There is also an increase in the fibrin yield.

The results of electrophoretic analyses are presented in Table III. Cold adaption resulted in decrease in albumin, and increase in β globulin and fibrinogen fractions. There also appears to be an overall increase in total protein under these conditions which although not statistically significant by these rigorous standards, was also present in preliminary studies. None of the serum patterns (results not presented) showed presence of any residual fibrinogen after clotting in either cold-adapted or normal animals.

[†] This concentration of CaCl_2 was used since it gave the shortest clotting time as determined in previous studies with rabbits.

[‡] Although the present data definitely suggests an increase in platelet count in cold-adapted animals, the statistical significance of the results might be questioned because of the poor reproducibility of this type of determination.

TABLE III. Relative Concentration of Proteins in Normal and Cold-Adapted Rabbits Based on Electrophoretic Data.

Rabbit No.	%					Total protein, g/100 ml
	Albumin	α -Globulin	β -Globulin	Fibrinogen	γ -Globulin	
Normal rabbits						
993	68.8	8.8	8.3	9.0	5.1	7.01
994	64.4	9.4	10.0	8.6	5.6	7.30
995	66.4	8.5	12.0	7.6	5.7	7.48
997	65.2	9.0	10.0	8.5	7.5	7.20
Mean	66.2	8.9	10.1	8.4	6.0	7.25
Cold-adapted rabbits						
977	58.6	8.4	14.8	11.8	6.3	8.03
978	53.7	8.0	15.8	14.0	8.7	7.18
979	61.0	6.6	13.4	12.5	6.6	7.30
980	56.0	8.2	13.6	15.0	7.2	7.93
982	57.6	9.5	13.5	11.6	7.9	7.38
Mean	57.4	8.1	14.2	13.0	7.3	7.56
Δ %*						
$3S_D$	2.08	.35	1.55	2.16	.33	.52

* Critical ratio—a value of 1 or greater indicates a probability of at least 99.7 that the means are significantly different.

Discussion. The observed increase in total protein (4.3%), erythrocyte count (5.9%), and hematocrit (7.4%) suggests that hemoconcentration occurred as a result of cold adaption. This effect has been observed by others (7,8) and evidence was presented which indicated that this phenomenon was of rapid development and short duration. Unfortunately we have no determinations of blood volume which would indicate whether the observed hemoconcentration was the result of reduced volume, or of increased quantities of the factors mentioned. Hemoconcentration, however, would not be expected to produce the observed changes in the clotting mechanism.

Increase in blood and plasma clotting time in the presence of decreased prothrombin time suggests that thromboplastin is less available in cold-adapted rabbits. Since there is an increase in platelet count it appears that platelets in these animals may be more resistant, although it is possible that thromboplastin is itself less effective for chemical reasons.

The decrease in prothrombin times in plasma from cold-adapted rabbits could be attributable to a number of factors, but may possibly be explained by increased fibrinogen shown by electrophoresis and increased fibrin yield. The prothrombin times are plotted in

Fig. 1 as a function of fibrin yield. Sufficient correlation exists between these parameters to indicate that at least part of the observed change is due to the increased fibrinogen.

Summary. Rabbits maintained for 2 months at 4°C, developed increases in platelet and erythrocyte count, hematocrit, plasma protein concentration, β -globulins and fibrinogen and in whole blood and plasma clotting times. Decreases occurred in serum albumin concentration and prothrombin time. It was suggested that retardation of clotting in spite of decreased prothrombin time might be due

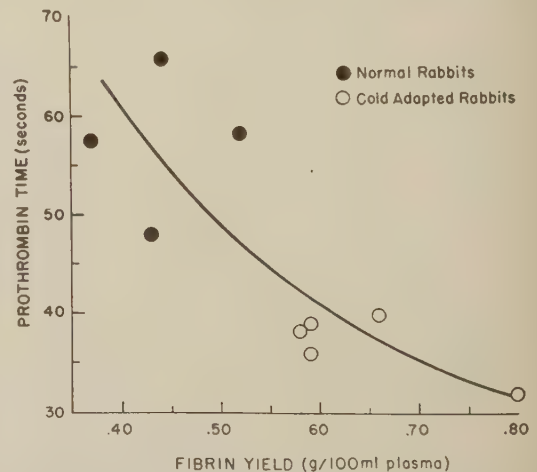


FIG. 1. Prothrombin times as a function of fibrin yield in all rabbits studied.

to an increase in resistance of platelets or to some chemical alteration of thromboplastin.

The authors gratefully acknowledge the technical assistance of Mr. Edward Henderson.

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Adrenal Corticoid Activities of 2-Methylhydrocortisone Acetate and 2-Methyl-9 α -Fluorohydrocortisone Acetate. (22171)

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Biological activity of hydrocortisone is markedly increased by halogenation of C-9 (1-5) and by dehydrogenation at positions C-1 and C-2 (6,7). When both modifications are present in the molecule, glucocorticoid, but not mineralocorticoid, activity of the steroid is further potentiated (8-10). This paper reports the results from biological testing of 2 new analogues of hydrocortisone: 2-methylhydrocortisone and 2-methyl-9 α -fluorohydrocortisone. These compounds have greatly enhanced gluco- and mineralocorticoid activities, as determined by subcutaneous administration on four different assays.

Materials and methods. Steroids. Steroid suspensions were made by grinding the compounds with carboxymethyl cellulose vehicle (8) in a ground glass tissue homogenizer. Compounds used were: 11 β , 17 α , 21-trihydroxy-4-pregnene-3,20-dione (F), 21-hydroxy-4-pregnene-3,20-dione, 21 acetate (DOCA), 9 α -fluoro-11 β , 17 α , 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (FF ac), 2-methyl-11 β , 17 α , 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (methyl F ac), and 2-methyl-9 α -fluoro-11 β , 17 α , 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (methyl FF ac). The latter 2 new steroids were recently reported by Hogg, Lincoln, Jackson and

Schneider (11). **Anti-Inflammatory Test.** This test measures the effect of steroids on granuloma formation in the rat by the cotton pellet assay described by Meier, Schuler and Desaulles (12) as modified by Dulin (9).

TABLE I. Anti-Inflammatory Assays Comparing Methylhydrocortisone Acetate, Methylfluorohydrocortisone Acetate, Hydrocortisone, and Hydrocortisone Acetate.

Compound*	Dose (mg/day)	No. rats	Mean granuloma dry wt (mg)	Potency ratio†
Vehicle	—	10	15	—
HA	.3	10	13.9	MA is
	.9	9	11.9	6.4 $\times$ HA
MA	.2	10	10.6	(3.4-10.7)
	.6	10	9.4	
Vehicle	—	8	14.2	
H	.3	8	11.7	MA is
	.9	8	8.4	4.5 $\times$ H
MA	.1	8	11.0	(2.6-7.2)
	.3	8	7.1	
Vehicle	—	10	15.3	
H	.2	9	10.6	MFA is
	1.0	8	6.3	9 $\times$ H
MFA	.02	9	9.4	(7.1-11.4)
	.1	8	7.3	

* HA = Hydrocortisone acetate; MA = Methylhydrocortisone acetate; H = Hydrocortisone; and MFA = Methylfluorohydrocortisone acetate.

† Figures in parentheses indicate limits of potency ratio at $P = .95$.

TABLE II. Comparisons of Methylhydrocortisone Acetate with Fluorohydrocortisone Acetate by the Liver Glycogen Technic.

Assay	Potency ratio* (methylhydrocortisone acetate/fluorohydrocortisone acetate)
1	.63 (.56-.71)
2	.97 (.80-1.17)
3	.85 (.65-.96)
4	.48 (.44-.54)
5	1.16 (.97-1.39)
Mean	.82

* Figures in parentheses indicate limits of potency ratio at $P = .95$.

Glycogen Deposition Test. The technic used was a modification(8) of the method of Pabst, Sheppard and Kuizenga(13). **Muscle Work Assay.** This assay was essentially that of Ingle(14,15) except that in one experiment electronic counters were substituted for mechanical work recorders. The steroids were injected in equal amounts twice in a 24-hour period. At the end of 24 hours, the experiment was terminated even though in some cases the rats were alive and still working. The work performed is reported in terms of revolutions of the counters. **Sodium Retention Test.** This test(8), a modification of the method of Marcus, Romanoff, and Pincus (16), measures the effect of compounds on sodium excretion in the adrenalectomized, salt- and water-loaded rat within four hours after administration. Since F is almost inactive on this assay(8), the reference standard

TABLE III. Glycogen Deposition Activity of Methylfluorohydrocortisone Acetate.*

Compound	Dose (μ g)	% liver glycogen
Hydrocortisone	300†	.5
	600	1.2
	900†	1.7
Methylfluorohydrocortisone acetate	5	.6
	10	.8
	15	1.3
	30	1.6
	45	1.9
	50	1.8
	90	2.4
	150	2.5

* Pooled data from 3 experiments.

† 10 rats/dose. All other values are means of 5 rat dosage groups. Calculated potency ratio is $38.0 \times$ hydrocortisone. Limits of error are 97-104%.

was DOCA. Data obtained from these assays were pooled and submitted to an analysis of potency ratios with respect to DOCA. Potency ratios in this and the preceding assays were calculated by the method of Irwin(17).

Results. Table I presents data from anti-inflammatory assays comparing methyl F ac with F and F ac, and methyl FF ac with F. The potency of methyl F ac is calculated at $6.4 \times$ F ac and $4.5 \times$ F, while methyl FF ac is approximately $9 \times$ F. By these same assay procedures, FF ac has been reported as being $7 \times$ F(9).

Potency ratios of methyl F ac to FF ac in the glycogen deposition assay are given in Table II. The value of .82 represents the

TABLE IV. Comparative Activity of Methylhydrocortisone Acetate, Methylfluorohydrocortisone Acetate, and Hydrocortisone on the Muscle Work Assay.

Treatment	Dose	Revolutions*	No. rats
Untreated controls	—	8244	34
Hydrocortisone	1 mg	20050	13
Methylhydrocortisone acetate	125 μ g	18630	16
	150	23280	14
	250	26500	4
	500	29680	4
Untreated controls	—	3895	10
Hydrocortisone	150 μ g	6624	14
Methylfluorohydrocortisone acetate	10 μ g	6247	13
	15	7921	10
	50	10427	6

* Mechanical counters were used in assaying methylhydrocortisone acetate, and electronic counters for methylfluorohydrocortisone acetate.

mean of 5 quantitative assays. Since FF ac has been found to be $13 \times$ F by this assay(8), the potency of methyl F ac can be calculated to be approximately $10 \times$ F. In Table III are presented data which indicate that methyl FF ac is $38 \times$ as potent as F on this test.

In the muscle work test, methyl F ac was $6-7 \times$ as potent as F (Table IV). Between this assay and the evaluation of methyl FF ac, electronic counters were substituted for mechanical calculators on the Ingle Fatigue Apparatus and, therefore, the values obtained on the latter assay cannot be directly compared with those presented for methyl F ac. The data obtained from comparing methyl

TABLE V. Sodium Retention Activity of Methylhydrocortisone Acetate and Methylfluorohydrocortisone Acetate as Compared with Desoxycorticosterone Acetate.

Compound	Dose (μ g)	No. rats	Sodium excretion (% of control)	Potency* ($\times$ DOCA)
DOCA	1	24	69	1.0
	2.5	6	59	
	5	12	48	
	10	126	48	
	20	12	35	
	40	12	27	
Methylhydrocortisone acetate	.44	6	90	2.6 (2.16-3.10)
	.88	6	68	
	1.0	12	68	
	1.5	6	78	
	1.75	6	57	
	2.0	12	52	
	3.0	6	42	
	3.5	6	46	
	4.0	12	41	
Methylfluorohydrocortisone acetate	6.0	6	34	
	.001	6	87	90.0 (68-123)
	.01	18	79	
	.1	24	41	
	1	18	24	
	10	6	24	

* Figures in parentheses indicate limits of error at $P = .95$.

FF ac with F on this assay are also presented in Table IV and indicate that this steroid has between 10 and 15 $\times$ the potency of F.

Comparison of potencies of methyl F ac and of DOCA by the sodium retention assay was difficult. Variation in results with methyl F ac made it necessary to conduct 5 multi-dose assays of this steroid. These data are presented in Table V. Methyl F ac is calculated at about 2.6 $\times$ DOCA. This steroid, therefore, has less mineralocorticoid activity than FF ac, which has been found to be 5 $\times$ DOCA on this assay(8). Data from assays of sodium retention activity of methyl FF ac are also presented in Table V and reveal the remarkable potency of this compound. Statistical analysis of the data shows methyl FF

ac to have approximately 90 $\times$ the sodium-retaining potency of DOCA.

Discussion. Methylation of F at C-2 increases the mineralocorticoid and anti-inflammatory properties of this hormone to a lesser extent than fluorination at C-9, but these two activities are in about the same ratio in methyl F ac and FF ac. This is in contrast with the Δ^1 analogue of F in which the anti-inflammatory potency is increased out of proportion to the electrolyte activity(9).

The glycogen deposition activity of FF ac is 13 $\times$ F and that of 11 β , 17 α , 21-trihydroxy-1, 4-pregnadiene-3,20-dione, 21-acetate (Δ^1 F ac) is 3 $\times$ F; both changes in the molecule (Δ^1 FF ac) resulted in this activity being 49 $\times$ F(8). The anti-inflammatory effect of

TABLE VI. Comparative Corticoid Activities of Methylhydrocortisone, Fluorohydrocortisone and Methylfluorohydrocortisone Acetates.

Assays	Steroids assayed		
	Methylhydrocortisone acetate	Fluorohydrocortisone acetate	Methylfluorohydro- cortisone acetate
Anti-inflammatory	4.5 $\times$ hydrocortisone	7 $\times$ hydrocortisone*	9 $\times$ hydrocortisone
Glycogen deposition	10 "	13 " †	38 "
Muscle work	6-7 "	not determined	10-15 "
Sodium retention	2.6 $\times$ DOCA	5 $\times$ DOCA †	90 $\times$ DOCA

* Dulin(9). † Stafford *et al.*(8).

FF ac is 7 x F and that of Δ^1 F ac is 3 x F; the Δ^1 -9 α -fluoro analogue has 14 x the potency of F(9).

In these experiments, a methyl group at C-2 and fluorine at C-9 in the hydrocortisone molecule did not cause as great a potentiation of glucocorticoid properties as both Δ^1 and fluorine in the same steroid nucleus. Regarding mineralocorticoid potency, however, quite the opposite is the case. Δ^1 F, like F, is almost inactive on the sodium retention assay used to evaluate these corticoids, and the Δ^1 -9 α -fluoro analogue of F has no more mineralocorticoid activity than FF ac(8). Whereas substitution of fluorine for hydrogen at C-9 and methyl for hydrogen at C-2 each causes a great increase in sodium retention activity of F, the tremendous mineralocorticoid potency of methyl FF ac is considerably greater than would be expected on the basis of the electrolyte activity of methyl F ac or FF ac.

Summary. (1) Two new analogues of hydrocortisone, 2-methylhydrocortisone acetate and 2-methyl-9 α -fluorohydrocortisone acetate, have been tested for corticoid activity on the following assays: anti-inflammatory, glycogen deposition, muscle work, and sodium retention. A summary of the data is presented in Table VI. (2) The sodium-retaining activity of 2-methyl-9 α -fluorohydrocortisone acetate is 90 x desoxycorticosterone acetate and is, therefore, one of the most potent mineralocorticoids.

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Effect of Ultraviolet Radiation on Iron-binding Capacity of Plasma. (22172)

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In applying our recently developed methods for the determination of plasma iron(1) to ultraviolet-irradiated plasmas (pooled blood-bank ACD plasmas, "UVR-sterilized"), we found significantly lower values for the iron-binding capacity of these plasmas compared with non-irradiated samples. The present

study was initiated to investigate this finding and to determine the extent to which siderophilin, the protein responsible for the specific iron-binding capacity of plasma(2,3) was affected by UVR.

Materials and methods. Plasmas obtained from blood-bank ACD-treated blood samples

TABLE I. Alteration of Siderophilin Iron-Binding Capacity with Irradiation.

Test material	UVR time (sec)	TIBC* ($\mu\text{g Fe/ml}$)	% TIBC remaining	$C \dagger \times 10^2 \times \text{sec}^{-1}$
(a) ACD plasma— $a \dagger = 67$, BI $\S = 0.98 \mu\text{g Fe/ml}$	0	2.53	100	
	25	2.06	81.5	.82
	50	1.57	62.0	.95
	75	1.46	57.7	.73
	100	1.35	53.4	.63
	150	1.31	51.8	.44
(b) Fraction IV-7— $a = 10.1$, BI = $0.26 \mu\text{g Fe/ml}$	0	3.91	100	
	10	3.03	77.5	2.5
	25	2.23	57.0	2.2
	50	1.37	35.0	2.1
	100	.86	22.0	1.5
	150	.84	21.5	1.0
(c) ACD plasma-Fraction IV-7 mixture— $a = 56.7$, BI = $0.29 \mu\text{g Fe/ml}$	0	3.04	100	
	15	2.59	85.2	1.1
	25	2.22	73.0	1.3
	50	1.70	55.9	1.2
	100	1.41	46.3	.77

* TIBC = Total iron binding capacity of 1 ml of test solution.

$$\dagger C = \frac{2.3}{t} \log \frac{\text{TIBC at } t = 0}{\text{TIBC at } t}$$

$$\dagger a = \frac{2.3 \times \text{optical density}}{\text{Light path, cm}} \times \text{dilution. Optical densities of appropriate dilution of test solutions of 1 cm depth were determined at } 254 \text{ m}\mu \text{ with a Beckman spectrophotometer.}$$

$\S$ BI = Bound iron contained in 1 ml of test solution.

|| These early values (0-20% siderophilin inactivation) are the least reliable since they are most affected by experimental error involved in determination of TIBC.

(1.84 g dextrose, 1.65 g sodium citrate, 0.6 g citric acid in 75 ml of water added to 500 ml of blood) and concentrated siderophilin, (plasma fraction IV-7, Cohn*), assaying 41% on the basis of its specific iron-binding capacity, were employed in this study. The irradiations were performed with an apparatus $\dagger$ having 2 Westinghouse cold-cathode Sterilamps as the UV light source. Their emission at 2537 Å represents approximately 95% of the total UV output. A glass-bottomed, plastic-sided tray (31 x 6 x 1.3 cm) was centered 2.5 cm below the outer surface of the lamps and parallel to their longitudinal axes. The average integrated radiation intensity at this distance was 11.3 ± 1.1 milliwatts/cm ^{2} as measured by the uranyl-oxalate actinometric method(4). A 3-ml sample of the test

solution was uniformly spread over the tray to a constant depth of 0.016 cm and exposed directly to the UV lamps at room temperature for exposure periods ranging from 10 to 150 seconds. Following the irradiation, each sample was removed from the tray and tested for its total iron-binding capacity (TIBC). The TIBC is a measure of the active siderophilin concentrations in plasma and represents the sum of both the iron-complexed and iron-free siderophilin species as determined by the bound iron (BI) and the unsaturated iron-binding capacity (UIBC) test methods, respectively(1). A decrease in the TIBC was considered a measure of siderophilin inactivation.

Results. Pooled plasma, fraction IV-7, and various plasma fraction IV-7 mixtures were irradiated according to the procedure described and their total iron-binding capacities determined. Table I presents the data obtained from typical experiments. It illustrates the quantitative relation between the amount of UVR received by the test siderophilin con-

* Courtesy of Dr. D. M. Surgenor, Harvard University, Boston, Mass.

$\dagger$ We are indebted to Dr. John T. Tripp of the Laboratory of Biologics Control, NMI, for the use of this apparatus and for generous donations of various plasma samples.

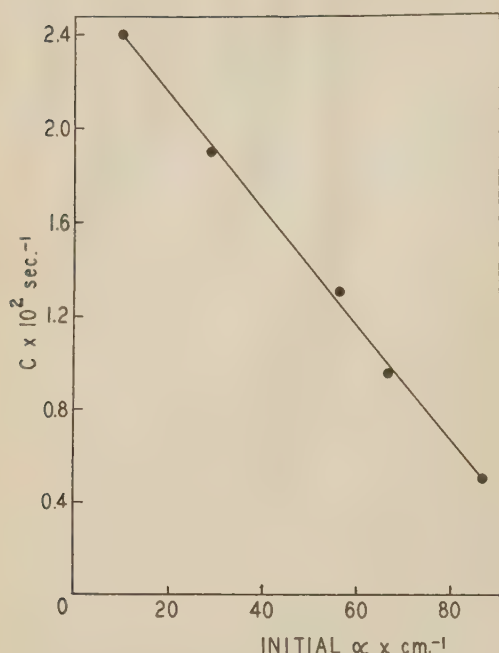


FIG. 1. Relation between rate of inactivation of siderophilin and absorption coefficient of the test solution.

taining solution and the decrease in its TIBC. The C values listed are the first order rate constants calculated on the basis that the effective radiation intensity was constant. It will be observed, however, that the absolute values of C are different for each of the several siderophilin solutions tested and that throughout the period of exposure of any one solution, the C values changed. Hence, these values are not true rate constants.

The magnitude of the C value for a given test solution is dependent upon the absorption coefficient (α) of that solution—the higher the α value the lower the C value. The relationship between C and α is illustrated in Fig. 1 in which the inactivation rates are plotted for solutions containing 2-3 mg siderophilin per ml and exhibiting a range of initial α values from 10.1 to 87. The solutions of intermediate α values were prepared by the appropriate dilutions of ACD plasma with the fraction IV-7 solution. The apparent rate constants were obtained by determining the time required for 50% inactivation ($t_{1/2}$)

from a plot of $\log \frac{\text{TIBC at } t = 0}{\text{TIBC at } t}$ versus time

t (seconds) and substituting the derived $t_{1/2}$ in $C = \frac{0.693}{t_{1/2}}$. It was necessary to extrapolate the linear plot to obtain the $t_{1/2}$ in cases where deviation from linearity occurred before this point was reached. The progressively decreasing rate of inactivation of siderophilin in the test solutions of increasing α value can be ascribed to the enhanced "internal screening" (5) of the effective radiation by the non-siderophilin plasma proteins.

The fact that the value of C did not remain constant during the period of irradiation of any one solution suggested that its initial α value changed during the exposure period. Experiments to elucidate this point showed that the α value of a given test solution did increase linearly for at least the first 50 seconds of exposure, *e.g.*, a plasma sample of initial α value of 67 increased 0.125 α unit/second while a fraction IV-7 solution with an initial α of 10.1 increased 0.11 α unit/second. For longer periods than 50 seconds, additional factors probably played a role in determining the amount of radiation absorbed. One such factor was the progressive formation of a visible surface film of coagulated protein clearly evident after 150 seconds exposure.

Since approximately one-third of the siderophilin present in normal plasma is iron saturated, experiments were performed to determine whether there was any difference in sensitivity to UVR of the iron-saturated and iron-free siderophilin in blood plasma. Table II illustrates the UV inactivation of siderophilin-enriched plasma solutions, 25 and 100% iron-saturated. The inactivation of the iron-saturated sample was measured by determination of the iron dissociated from the complex under conditions comparable to those used for determining the unsaturated iron-binding capacity (UIBC). Subject to the limitations of the methods employed, the inactivation rates of the iron-free and iron-saturated siderophilin species were the same.

Summary. 1. Ultraviolet irradiation of ACD plasma and of plasma fraction IV-7 solutions inactivates the siderophilin component as measured by the loss of its specific iron-binding capacity. 2. The initial inactivation

TABLE II. Effect of UVR on Iron-Saturated and Iron-Unsaturated Plasma Siderophilin.

UVR time (sec)	Iron-saturated—100%			Iron-free—74.4% Iron-saturated—25.6%		
	TIBC, μg Fe/ml	% TIBC	$C \times 10^2 \times \text{sec}^{-1}$	TIBC, μg Fe/ml	% TIBC	$C \times 10^2 \times \text{sec}^{-1}$
0	3.94	100		3.94	100	
25	2.99	75.9	1.10	2.93	74.5	1.18
50	2.61	66.3	.82	2.76	70	.71
100	2.16	54.8	.60	2.06	52.3	.65
150	1.82	46.2	.52	1.93	49.1	.47

rates, whose magnitude was correlated with the absorption coefficients of the irradiated siderophilin solutions, followed essentially first order kinetics. 3. The UV inactivation rates of iron-free and iron-saturated siderophilin were found to be the same.

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Effect of New Adrenal Steroids on Electrolyte and Water Excretion. (22173)

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Recent clinical work has indicated that the Δ^1 -analogues of hydrocortisone and cortisone, namely prednisolone and prednisone, are in certain respects more potent than the parent compounds and differ from them qualitatively in that they cause little or no sodium and water retention at therapeutically effective doses (Bunim, *et al.*, 1, 2; Spies, *et al.*, 3; and others). Pechet and Bartter(4) found that in Addisonian patients prednisone would cause sodium loss whereas cortisone caused sodium retention. This action was correlated with a marked ability of the Δ^1 -compounds to increase the glomerular filtration rate. In animal studies, Perlman and Tolksdorf(5) found the Δ^1 -steroids more potent than their natural counterparts in causing liver glycogen deposition, eosinopenia and life-maintenance, but their effects on electrolyte excretion were unchanged. In tests designed particularly to measure sodium-retaining potency, Stafford, *et al.*(6) found pred-

nisolone to have "very slight" activity.

It is well known that adrenal cortical hormones are necessary for the maintenance of a normal rate of water diuresis and that deficiencies in this respect are repaired effectively by hydrocortisone and cortisone, but weakly, if at all, by desoxycorticosterone. We considered it of interest to determine, therefore, the relative activity of hydrocortisone, desoxycorticosterone (DC), prednisone* and prednisolone in this test. Excretion of sodium and potassium was measured also in the water-loaded test animals. Under the experimental conditions used, hydrocortisone and cortisone are known to increase sodium and potassium as well as water excretion.

Methods. Fasted male rats weighing approximately 200 g were tested 18 hours after adrenalectomy. At the beginning of an ex-

* Prednisone (Meticorten) and prednisolone (Meticortelone) were generously supplied to us by Schering Corp.

EFFECT OF CORTICOIDS ON WATER AND ELECTROLYTE EXCRETION

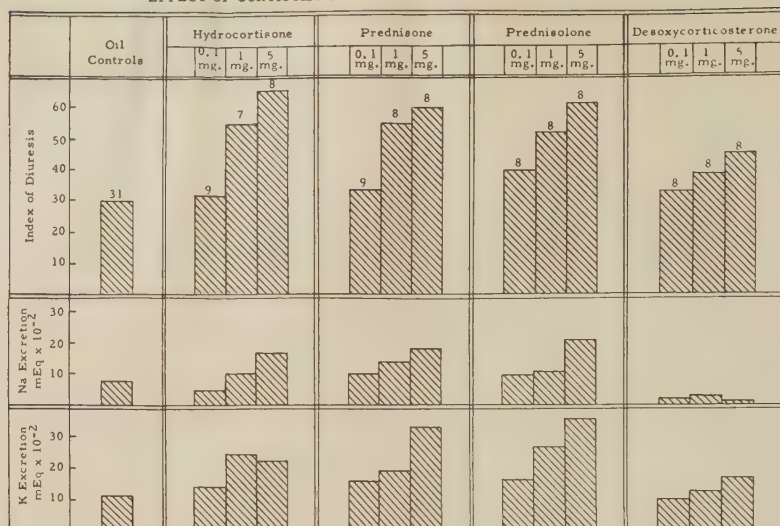


FIG. 1. Effect of various corticoids on water, sodium and potassium excretion over a 5-hr period in water-loaded adrenalectomized rats. Numbers above columns indicate number of animals on which mean values are based. "Index of Diuresis" represents in cm^2 the area under cumulative water excretion curves: increasing numbers represent increasing rates of water excretion.

periment, they were given 2 doses of distilled water ($3 \text{ ml}/100 \text{ cm}^2$ — ref. 7) an hour apart by stomach tube. The rate of water excretion was followed at half-hour intervals for 5 hours, starting from the first water load, and the total amounts of sodium and potassium excreted in that time were determined by flame photometry.

To express the effect of treatment on water excretion as one figure, the method of Krieger and Kilvington(8), as used by Ham and Landis(9), was employed: for each rat the rate of cumulative water excretion, expressed as per cent total load given, was plotted for the 5-hour period. The scale of the graph was such that 1 cm on the ordinate equaled 10% excretion of the total water load and 1 cm on the abscissa equaled 30 minutes time. The area under each curve was measured with a planimeter and the region encompassed in sq. cm designated the *Index of Diuresis*. Steroids were administered in oil, either in solution (small doses) or partial suspension (large doses) in amounts of 0.1 mg to 5 mg/rat. One-half of the total dose was injected subcutaneously at the time of adrenalectomy and one-half 30 minutes before the first water load. Control rats received sesame oil.

The significance of possible differences between the different treatments was tested primarily by analyses of variance (Snedecor, 10).[†] Since the nature of the results is in most respects self-evident from the graphs, statistical details are not included here.

Results. 1. Water Excretion. All of the steroids tested enhanced the sluggish rate of diuresis which characterizes adrenal insufficient animals and man (Fig. 1). In this respect no clearly significant differences were observed between the action of hydrocortisone, prednisone and prednisolone at any dose level. All 4 of the compounds tested gave dose-response curves with essentially parallel slopes. The response to DC, however, was definitely weaker ($P = < .01$) than any of the other compounds. None of the steroids was clearly effective at $100 \mu\text{g}$ dose levels.

2. Potassium Excretion. All 4 compounds increased potassium excretion (Fig. 1). Again DC was the weakest of the 4 and consistently active only at 5 mg doses.

3. Sodium Excretion. The only qualitative difference observed between the 4 compounds

[†] The authors are indebted to Mr. James Webster for statistical analysis of these data.

was that DC caused sodium retention and the other 3 steroids enhanced sodium excretion (Fig. 1). The 3 natriuretic compounds were equi-potent.

Discussion. It has been repeatedly demonstrated that the action of adrenal cortical steroids on water and electrolyte excretion can be altered or reversed depending upon a number of variables, some of which are undefined. For instance, hydrocortisone may cause either sodium retention or sodium diuresis when used in different rat tests specifically designed to detect and measure sodium-retaining activity. In other words, the response to the steroid is determined in part by intrinsic properties of the steroid itself, but equally or more so by the physiological environment with which it is provided. The present results and those of Perlman and Tolksdorf(5) suggest that those alterations in sodium-retaining properties of certain steroids resulting from the presence of a 1-2 double bond do not alter their ability to enhance sodium excretion under conditions where such activity might be expected.†

From all of the work now available it is apparent that the Δ^1 -steroids are, relative to their parent compounds, weaker in certain respects (*e.g.*, sodium retention in clinical use), stronger in several properties (anti-inflammatory effects, etc.) and of unchanged potency in others (water diuresis, etc.).

Summary. The effects of prednisone, prednisolone, hydrocortisone and desoxycorticosterone on excretion of water, sodium, and potassium, were compared in water-loaded adrenalectomized rats. All steroids stimulated water diuresis and potassium excretion. Hydrocortisone, prednisone and prednisolone were equi-potent; desoxycorticosterone was weaker. Hydrocortisone, prednisone and prednisolone stimulated sodium excretion at all doses having any effect and were again approximately equi-potent. Desoxycorticosterone had the opposite effect and caused sodium retention. The relative inability of the Δ^1 -steroids to cause sodium retention under conditions of clinical use is, therefore, not associated with any comparable inability to cause natriuresis under appropriate circumstances.

† In addition, the authors have found (unpublished) that hydrocortisone, prednisone and prednisolone have about equal potency in antagonizing the antidiuretic effect and enhancing the natriuretic effect of exogenous vasopressin.

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Progestational Activity of Halogenated Corticosteroids and Related Compounds in Rabbit and Monkey.* (22174)

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Fried(1) has reviewed the biological effects of the 9 alpha halo derivatives of hydrocortisone, cortisone, 11-beta-hydroxyprogesterone and other related steroids. The high mineralocorticoid and glucocorticoid activity shown by several of these compounds in experiments with rats and dogs were described. Results of the present investigations demonstrate progestational activity of several of the 9 alpha halo steroids in both the rabbit and monkey.

Materials and methods. *Clauberg rabbit test.* Female New Zealand white rabbits weighing 600-800 g at the beginning of treatment were used in all experiments. Ten μ g of estradiol 17-beta were given daily subcutaneously in 0.25 ml sesame oil solution for 6 days. Beginning on the 7th day, the test compounds were given subcutaneously daily for 5 days. The animals were killed with chloroform 20 to 24 hours after the last injection. Body, uterus and adrenal weights were recorded. Uteri and adrenals were fixed in Bouin's solution. Sections were stained with hematoxylin and eosin. Progestational proliferation of the endometrium was measured according to the standard scale of McPhail (5). *Inhibition of estrogen withdrawal bleeding in the monkey.* Bilaterally ovariectomized female monkeys (*Macacus rhesus*) weighing 3.5-5.5 kg were given daily subcutaneous injections of 10 μ g estradiol 17-beta in 0.25 ml sesame oil solution for 20 days. Subcutaneous injections of the test compound were begun on the 21st day. Daily vaginal smears were made with normal saline. Treatment was discontinued either with the onset of menstrual bleeding or when the experiment was terminated in order to obtain samples of the endometrium. With the exception of progesterone

and desoxycorticosterone acetate (in solution), all compounds were administered as suspensions in sesame oil.

Results. It is well to keep in mind the quantitative variability of the bioassay method when the potency of compounds is compared by means of the Clauberg rabbit assay. We have chosen a total dose of 0.5 mg progesterone as the reference level because this amount will produce full progestational proliferation of the endometrium in more than 75% of the rabbits receiving this dose whereas 0.25 mg progesterone gives a maximum progestational response in 28% of animals on this lower dose. These percentages were derived from a total of 61 rabbits on the 0.5 mg dose and 22 rabbits on the 0.25 mg dose.

Data from several experiments are presented in Table I. It will be noted that at a total dose of 5 mg, the activities of 9 alpha-fluoro-hydrocortisone acetate, 9 alpha-chloro-hydrocortisone acetate and desoxycorticosterone acetate are comparable whereas hydrocortisone acetate is inactive even at a level of 25 mg. At a total dose of 15 mg, both the 9 alpha-fluoro- and 9-alpha-chloro derivatives of hydrocortisone produce a response comparable to that of 0.5 mg of progesterone (full proliferation). This would indicate an activity for these halo compounds of about 3% that of progesterone.

Delta-1-9-alpha-fluorohydrocortisone acetate was also assayed by the Clauberg method. This compound gave minimal progestational responses both at total doses of 10 mg and 20 mg.

Table I includes results on progestational proliferation produced in response to treatment with 9-alpha-fluoro-11-beta-hydroxyprogesterone. A 1.0 mg total dose of this halogenated compound possesses low grade activity and at the 3.0 mg level there is a moderate response. At 5.0 mg a maximal response was elicited. An estimated potency of 5 to 10% that of progesterone could be made for this

* The 9 alpha-halo steroids used in this study were supplied through the courtesy of Dr. J. Fried of the Squibb Institute for Medical Research. The delta-1 steroids were supplied through the courtesy of Dr. Marvin Kuizenga of the Upjohn Co. and Dr. Preston Perlman of the Schering Corp.

TABLE I. Progestational Activity of Halogenated Steroids in the Clauberg Assay.

Total dose (mg)	Progestational proliferation					
	9 alpha-chloro F acetate	9 alpha-fluoro F acetate	Cpd. F acetate	DOC acetate	9 alpha-fluoro 11-beta-hydroxyprogesterone	Progesterone
25			0, 0, 0			
15		4, 3, 4 4, 4, 4				
10	4, 4, 4*	3, 2, 2 3, 3, 2	0, 0, 0	4, 4, 3	4, 4	
5	3, 4, 4, 1, 2 2, 2, 1 2, 1, 1	2, 1, 1 1, 1, 1	0, 0, 0	2, 1, 1	4, 4	
3.0					3, 2	
2.5	0, 0, 0 0, 0, 0	0, 0, 0	0, 0, 0	2, 2, 1		
1.0	0, 0, 0	0, 0, 0			2, 2, 1	
.5	0, 0, 0					4, 4, 4 3, 4
.25						4, 4, 3, 3, 3

* Each number represents degree of progestational proliferation for one animal.

halo steroid. Neither delta 1-hydrocortisone nor delta 1-cortisone showed progestational activity when tested at a total dose of 20 mg using 3 animals for assay of each compound. Cortisone acetate was inactive as a progestogen at a total dose of 50 mg (3 rabbits).

Gross examination of animals receiving the 10 and 15 mg doses of fluoro- and chlorohydrocortisone and the 25 mg dose of hydrocortisone revealed ascites and liver enlargement. Adrenal weights were consistently

lower in these animals as compared with those on progesterone treatment.

Results obtained from experiments with estrogen-primed castrate female rhesus monkeys are shown in Fig. 1. Three untreated monkeys began estrogen withdrawal bleeding on the fifth day following the last injection of estradiol. The onset of bleeding was delayed from 9 to 24 days in 3 monkeys receiving 1.0 mg progesterone daily. Of 2 monkeys treated with a daily dose of 40 mg of 9 alpha-fluorohydrocortisone acetate, one continued for 9 and the other for 18 days without withdrawal bleeding. Treatment of these animals was discontinued to permit uterine biopsies. Histological examination of the biopsies presented a clearly defined progestational endometrium. In another animal, a daily dose of 20 mg of 9 alpha-fluorohydrocortisone acetate failed to delay estrogen withdrawal bleeding.

Our results demonstrated progestational activity of 9 alpha-chlorohydrocortisone, 9 alpha-fluorohydrocortisone and 9 alpha-fluoro-11-beta-hydroxyprogesterone in the rabbit. Hydrocortisone was shown to be inactive even at high levels. Delay of estrogen withdrawal bleeding in the monkey further demonstrates the progestational action of 9 alpha-fluorohydrocortisone. The lack of toxicity and any

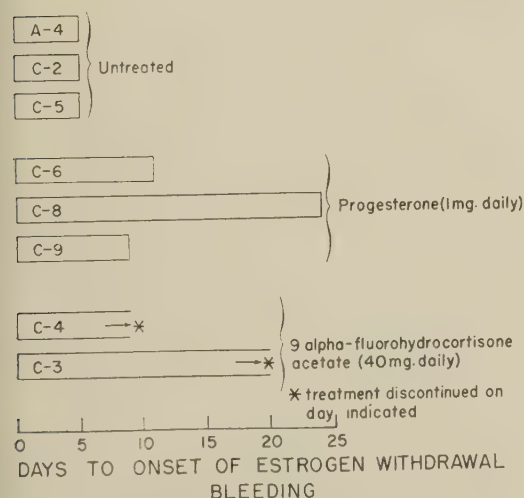


FIG. 1. Progestational activity of 9 alpha-fluorohydrocortisone acetate in the monkey.

unusual side effects of this halogenated corticosteroid in the monkey is in marked contrast with effects noted in the rabbit.

Discussion. Byrnes and Shipley(2) reported a lack of progestational activity for 11 beta-hydroxyprogesterone at a dose of 5 mg in the Corner-Allen assay. It is evident that the introduction of either a chlorine or a fluorine atom in the 9 alpha position imparts progestational activity in hydrocortisone and 11-beta hydroxyprogesterone both of which were progestationally inactive at comparable doses.[†] Junckmann(3) has reported that 17 alpha-hydroxyprogesterone, slightly active as a progestational compound, becomes active upon esterification. Huggins and Jensen(4) have recently described the potent depressant activity of 9 alpha-fluorohydrocortisone and 9 alpha-11-beta-hydroxyprogesterone on estrone-induced uterine growth in the hypophysectomized rat. Hydrocortisone and cortisone were considered to be weak inhibitors in the

[†] However, 11-beta-hydroxy progesterone has one-eighth the activity of 9 alpha-fluoro-11-beta-hydroxy progesterone in the Clauberg Test.

same test.

Summary. Progestational activity of 9 alpha-chlorohydrocortisone acetate, 9 alpha-fluorohydrocortisone acetate and 9 alpha-fluoro-11-beta-hydroxyprogesterone has been demonstrated using progestational proliferation of the rabbit endometrium (Clauberg test) as index of activity. Hydrocortisone acetate and cortisone acetate show a lack of activity in the same test. Progestational activity of 9 alpha-fluorohydrocortisone is further demonstrated by prevention of estrogen withdrawal bleeding and characteristic histological changes in the endometrium of the ovariectomized rhesus monkey pre-treated with estrogen.

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Metabolism of the Lanthanons in the Rat.* (22175)

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For many years the behavior of the lanthanons in biological systems was regarded as a scientific curiosity. The name "lanthanon" has been suggested(1) for the group of elements of atomic numbers 57 to 71, lanthanum through lutetium. Until recently the commercial use of these elements was limited and their chemistry relatively obscure. With the development of the chain-reacting pile, and the discovery that certain light members of this group were produced in relatively large quantities in the fission process, knowledge of their metabolism[†] and radiotoxicity as-

sumed greater importance. Certain members of this group can be reduced to the divalent state and others oxidized to the quadrivalent state; the principal valence state of the group as a whole, however, is plus three. Although the actual radius of an ion of a particular element depends principally upon its valency, the radii of the trivalent ions of the lanthanons show an interesting decrease with increasing atomic number. This phenomenon

[†] There has been much dissension concerning the use of the word "metabolism" for elements not intimately involved in biological processes; this terminology, however, is in general use in tracer work of the type presented here.

* This work was performed under auspices of Atomic Energy Commission.

has been called the "lanthanide contraction." (2) As the ionic radius decreases, the basicity of the lanthanons decreases and their compounds become more soluble, which may, to some extent, account for the differences in distribution of the light and heavy lanthanons described here. A complete review of the history, occurrence, applications, and chemistry of the lanthanons has been published by Vickery(3). The relationship of the lanthanons to biological systems was studied almost as soon as the first members of the group were isolated. A complete bibliography to 1935 on the biological occurrence, metabolism, and toxicity of the lanthanons has been prepared (4). In many biological specimens traces of cerium and lanthanum were found. A survey of the experimental exposure of living systems to lanthanons indicates that in vertebrates the toxic effects following parenteral injection were probably due to the formation of insoluble phosphates and carbonates, which disturb the hydrogen ion concentration of the organism. The observation was frequently made that the lanthanons were nontoxic (even in large amounts) when given orally. This was probably because they are poorly absorbed from the gastrointestinal tract. Under the auspices of the Manhattan Project, tracer studies were performed in this laboratory(5,6) with some of the lighter members of the lanthanon series that are produced in nuclear fission. Some of these were carrier-free,[†] and others were administered with considerable amounts of stable isotope carrier, making direct comparisons difficult.

The studies reported here were designed to correct some of the defects of the earlier work and to expand the data to include all the lanthanons on an uniform and systematic basis. The recent availability of extremely pure specimens of the rarer lanthanons, and the construction of the high-neutron-flux Materials Testing Reactor, made possible the production of the necessary quantities of pre-

viously unobtainable radioisotopes of high specific activity. The age, sex, and the strain of the rats, and the care given them, were uniform throughout. The size of the groups was increased. The radioisotopes were administered as citrate complexes, which increased the speed of absorption from the site of injection.

Methods. Preparation of Radioisotopes. The radioisotopes of cerium, europium, and promethium were obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee. The praseodymium data are reprinted with the kind permission of Josephine Crowley-Ellis(7). Radioactive thulium and terbium were provided by Drs. Glenn T. Seaborg and Stanley Thompson of the University of California Radiation Laboratory. Dr. Frank Spedding of the University of Iowa, Ames, Iowa, very kindly supplied 99.9 to 99.95% spectroscopically pure oxides of the remaining lanthanons from which radioisotopes were prepared by the (n, γ)[§] reaction. Weighed portions of the oxides were diluted in 6 N HCl. Aliquots were transferred to clean quartz ampules. The ampules containing the lanthanon solutions were dried overnight in an oven at 105°C and heated at 200°C for a few minutes to expel all traces of water. They were then sealed and sent to Arco, Idaho, where they were placed in the Materials Testing Reactor in the region of maximum neutron flux. After bombardment, the materials were flown back to this laboratory. The ampules were placed in heavy plastic envelopes and crushed. The crushed ampule was transferred to a beaker and washed several times with 6 N HCl or until a survey meter showed that no more activity could be removed from the quartz. Ten milligrams of NaCl were added to the solution, which was evaporated to incipient dryness. Sodium citrate solution, 30 mg/ml, was added, the amount varying with the quantity of activity present, and the pH was adjusted to neutral with 9 N NaOH. The latter procedure—evaporating with salt and redissolving—was also followed with the isotopes obtained from

[†] A carrier-free preparation is one in which all the atoms present of a particular element are radioactive. The specific activity of such a preparation is by definition, $\frac{N \text{ active}}{N \text{ total}} = 1$.

[§] Dy¹⁶⁶ was prepared by a second-order reaction, Dy¹⁶⁴(n, γ), Dy¹⁶⁵(n, γ), Dy¹⁶⁶.

TABLE I. Summary of Time Intervals Investigated and the Amounts of Rare Earth, and Complexing Agent Administered per Rat.

Isotope	Time interval (days)	Dose (μc)	Carrier (μg)	Sodium citrate (mg)
La ¹⁴⁰	1, 4	29, 58*	1.2, 2.4	6, 12
Ce ¹⁴⁴	1, 4, 64, 256	20-40†	c.f.‡	3-6
Pr ¹⁴³	1, 4, 15, 32	180	c.f.	2
Nd ¹⁴⁷	1, 4, 16	2.3-4.6	1.1-3.7	2.7-5.6
Pm ¹⁴⁷	1, 4, 64, 238	10-70	c.f.	3-6
Sm ¹⁵³	1, 4	30, 75	0.3, 0.6	3, 7.5
Eu ^{152, 154}	1, 4, 64, 256	1.8-2.6	5.2-7.8	5.7-8.6
Gd ¹⁵⁹	1, 4	3.8, 20	2.0, 10.8	2.3, 12
Tb ¹⁶⁰	1, 4, 64, 256	3.0-12.0	3.0-12.0	2.8-11.2
Dy ¹⁶⁶	1, 4	4.3, 11.0	0.1, 0.3	3.0, 7.5
Ho ¹⁶⁶	1, 4	14, 84	0.02, 0.12	1.3, 7.8
Er ¹⁶⁹	1, 4, 16	2.4-4.8	1.0-3.8	2.8-5.6
Tm ¹⁷⁰	1, 4, 64, 256	3-12	0.08-0.3	3-12
Yb ¹⁷⁵	1, 4, 8	14-57	0.4-1.1	2.5-7.4
Lu ¹⁷⁷	1, 4, 16	7.3-29	0.5-1.9	4.8-7.2

* One- and four-day dosages respectively.

† Dosages graded for time intervals involved.

‡ c.f. = carrier-free.

other sources. The radioactive purity of all the preparations, and the identity of the isotopes were determined by the measurement of the half life, the beta-ray absorption in aluminum, and the gamma-ray absorption in lead. The specific activities were estimated from the weight of the bombarded sample and the total volume of the final citrate solution. The half lives, beta energies, and the presence or absence of gamma rays of the isotopes employed are listed in Hollander *et al.*(8).

Biological Procedures. The animals used in these tracer studies were female Sprague-Dawley rats that were from four to six months of age (200-250 g) when injected. The animals were maintained on tap water and a pelleted stock diet developed by the University of California Institute of Experimental Biology, "Diet 14," for at least two weeks prior to isotope administration and until sacrifice. While the animals were under light ether anesthesia, isotopes of all the lanthanons were administered intramuscularly in the left hind leg and Ce¹⁴⁴, Eu^{152, 154}, Tb¹⁶⁰, and Tm¹⁷⁰ were also given orally by stomach tube. In no case did the volume of solution administered exceed 1 ml. The time intervals investigated, the dosage of each radioisotope in microcuries per rat, the amount of

stable lanthanon per rat, and the amount of sodium citrate administered per rat are shown in Table I. Moderate amounts of high-specific activity preparations were used in order to avoid uncertainties introduced by either radiotoxicity or chemical toxicity. The largest amount of radioactivity administered, 0.5 $\mu\text{c/g}$ of body weight, was felt to be well below radiotoxic level(9). The amount of complexing agent used was roughly adjusted to the quantity of carrier present. The actual dosage for any given experiment, both in microcuries and micrograms, depended on the best balance obtainable among the following factors: specific activity, half life, counting accuracy desired, and in most cases, beta-particle energy. In the intramuscular studies at least 5 rats were injected for each time interval. In the oral studies, groups of three animals were used. A standard was prepared for each experiment; at the time of injection, an amount of the radioactive solution equal to that administered to each animal was diluted to 100 ml in 2 *N* HNO₃. Following administration of isotope, groups of 2 or 3 animals were placed in metabolism cages which were designed to provide adequate separation of urine and feces with a minimum of handling. Excretions were collected daily for the first 4 days after injection and twice weekly thereafter. In the intramuscular studies animals were sacrificed at intervals of one and 4 days after injection in all the experiments, and when the half life permitted, at longer time intervals ranging from 8 to 256 days. In the oral studies the animals were sacrificed at four days. Under chloroform anesthesia, a blood sample was withdrawn by heart puncture, after which the animals were returned to the chloroform for sacrifice. The left hind leg (the site of injection) was removed at the pelvic girdle, and the foot was cut off for assay with the carcass. The remaining carcass was then skinned, and the following tissues and organs were dissected and weighed: pelt, spleen, liver, kidneys, gastrointestinal tract, gastrointestinal contents, the muscle from the

|| An outbreak of pneumonia in the colony reduced the number of rats to four in the 256-day Eu^{152, 154}, and Tb¹⁶⁰ experiments.

right hind leg, and the femur, fibula, and tibia from the right hind leg.

Preparation of Samples for Beta Assay. The blood, spleen, kidneys, muscle, and gastrointestinal tract (small samples) were placed in individual porcelain ashing capsules, and the liver, bone, skin, gastrointestinal contents, left leg, carcass and excreta (large samples) were placed individually into beakers. All tissues were dried for 24 hours at 100°C and ashed for 24 hours at 500°C. The ash from the small samples was spread evenly by dissolving in 2 *N* HNO₃ and redrying. The soft-tissue ash in the carcass was removed from the skeleton by rinsing with distilled water and filtering through gauze. The skeletal ash was dried and weighed. The large samples, including skeleton, were dissolved in 2 *N* HNO₃, and aliquots were transferred to weighed porcelain capsules and dried. To correct for self-absorption of the beta particles in the mass of the sample, dilution curves were prepared for each isotope according to the methods previously described (10). The weights of the large samples were determined by difference. The weights of the small samples were estimated on the basis of 1.25% ash in soft tissues. An aliquot of the standard was assayed whenever samples were counted to eliminate corrections for decay and fluctuations in counter efficiency. A self-quenching Geiger-Muller counter was employed for the beta particle assays, and all samples were counted for a sufficiently long time to reduce the error of measurement to $\pm 5\%$ (11).

Preparation of Samples for Gamma Counting. Gamma rays were present in sufficient intensity for accurate measurement of La¹⁴⁰, Ce¹⁴⁴, Sm¹⁵³, and Yb¹⁷⁵. The procedures employed for preparation of the skeleton and excreta samples were the same as described in the previous section. Other tissues and organs were placed in tin bottle caps. The samples were assayed wet with a TlI-NaI scintillating crystal counter of the type described by Jenkins (12).

Calculations. The total amounts of radioisotopes present in blood and muscle were calculated on the basis that these two tissues

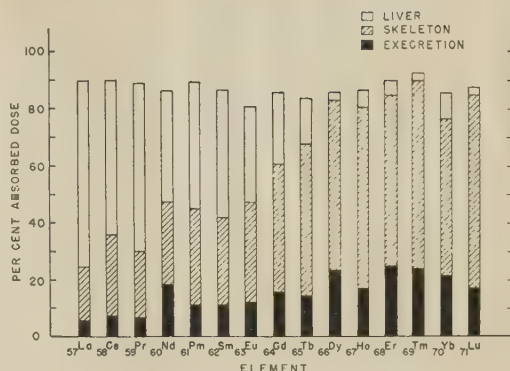


FIG. 1. Summary of the excretion, skeletal deposition, and liver deposition of the lanthanons one day after intramuscular administration. The difference between the end of each bar and 100% represents the % of each isotope in the remainder of the animal.

represent 7 and 45% of the body weight respectively. The lanthanon concentration in bone was calculated from the ash content of mature rat bone, $36.6 \pm 0.7\%$. The amount

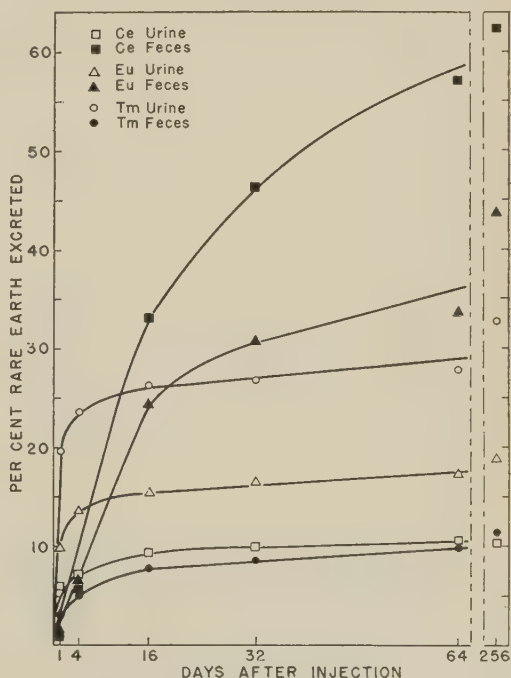


FIG. 2. The cumulative excretion of Ce¹⁴⁴, Eu^{152,154}, and Tm¹⁷⁰ in urine and feces. Values are corrected for deviation of recovery from 100% and are expressed as % of absorbed dose. The one-day points represent eight determinations, the four-day points six determinations, the 16-, 32- and 64-day points four determinations, and the 256-day points two determinations.

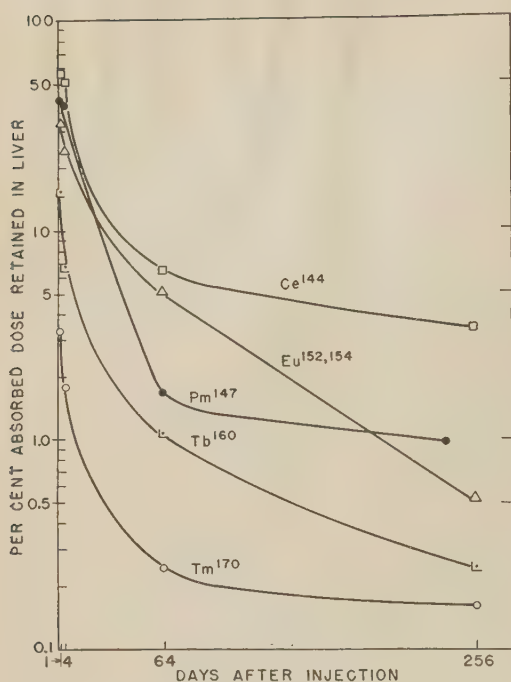


FIG. 3. The liver retention of Ce¹⁴⁴, Pm¹⁴⁷, Eu^{152,154}, Tb¹⁶⁰, and Tm¹⁷⁰. Each point represents five animals.

of each isotope remaining at the site of injection was calculated by subtracting the activity present in the muscle and bone samples from that found in the whole left leg. The residual per cent of dose, which has been designated "balance" consisted of the thoracic organs; reproductive organs; cartilage; glandular, lymphoid, connective and nervous tissue; fat; and blood vessels. All data are expressed as concentration and as the per cent of the absorbed dose found in the whole specimens, and are corrected for deviation of recovery from 100%.

Results. The trend of the results of the tracer studies with the fifteen lanthanons is represented in Fig. 1, which shows the per cent of administered dose in liver, skeleton and excreta one day after lanthanon administration. Fig. 2 shows the cumulative excretion of three representative lanthanons. The retention of five representative lanthanons in the liver and skeleton are shown in Figs. 3 and 4.

The experimental recoveries of the injected

material ranged from 86% for the 256-day Tm¹⁷⁰ to 121% for the 4-day Lu¹⁷⁷. Recoveries below 95% or greater than 105% were exceptional, however, indicating that on the whole the data are reliable.

Absorption. Absorption from the intramuscular injection site was, for the most part, fairly complete. In 11 of 15 experiments, the amount of isotope unabsorbed four days after injection was less than 6.5%. Three of the four exceptions, Eu^{152,154}, Gd¹⁵⁹, and Tb¹⁶⁰ (15% unabsorbed at 4 days), were administered with 8 to 10 μ g of carrier, the largest quantities employed. The ratio of micrograms carrier to milligrams sodium citrate was 0.9, also considerably higher than in most of the experiments. In the La¹⁴⁰ studies (22.5% unabsorbed at 4 days), the ratio of carrier to complexing agent was 0.2 μ g/mg. The insolubility of the compounds of lanthanum at physiological pH's is probably a major factor in its difficult absorption. The rate of absorption of the lanthanons from the intramuscular injection site seems to depend upon the solubility of their compounds, the amount

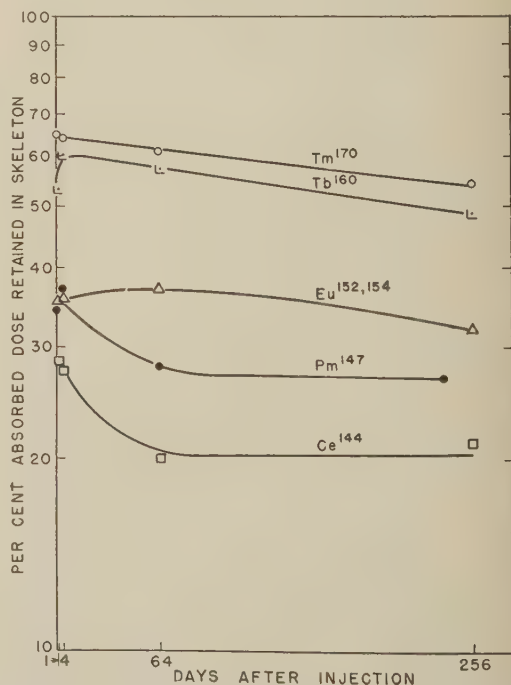


FIG. 4. The skeletal retention of Ce¹⁴⁴, Pm¹⁴⁷, Eu^{152,154}, Tb¹⁶⁰, and Tm¹⁷⁰. Each point represents five animals.

of stable carrier administered and, to some extent, upon the ratio of the quantity of carrier to the quantity of sodium citrate administered. Absorption of Ce^{144} , $\text{Eu}^{152,154}$, Tb^{160} , and Tm^{170} from the gastrointestinal tract was less than 0.1% of the administered dose. Because of the great mass of the data, only significant trends will be discussed.†

Spleen. The concentration of the lanthanons in the spleen one day after administration was less than 0.5% per g of wet tissue. When these elements are administered by vein as halides or nitrates, high concentrations are found in the spleen as well as in other components of the reticulo-endothelial system. Laszlo *et al.*(13) concluded that they were dealing, to a large extent, with radio-colloids. Bruner *et al.*(14) in studies with radiogallium (principal valence plus three) reached similar conclusions. The intramuscular route was chosen in the present experiments in order to minimize the formation of insoluble lanthanon colloids in the blood stream. The low spleen concentrations demonstrated that this aim was largely achieved; that these data represent the metabolism of these elements in non-colloidal form.

Blood, Muscle, and Skin. The level of these elements in the blood at one day never exceeded 0.02% per ml, and there was a steady decrease to unmeasurable levels thereafter. The initial muscle concentration of the lanthanons was the same as for blood, but declined at a somewhat slower rate. The skin concentrations throughout the series, at the early time intervals, were about three to four times as great as those found for muscle and blood, subsequently diminishing at about the same rate as did the concentrations in muscle. It is possible that at the early time intervals there may have been some contamination of the skin by the urine. This would apply particularly to the heavy members of the series, for which the urine is the main route of excretion.

Gastrointestinal Tract. The gastrointestinal tract generally showed a concentration about ten times that of blood and muscle at

the earlier time intervals. Two to eight months after administration the gastrointestinal concentrations had dropped to low but still measurable levels in the lighter half of the series, for which the gastrointestinal tract is the chief excretion route, and to unmeasurable levels in the latter half of the series. Retained fecal matter, particularly in the cecum, may have contributed to the initial high concentrations in the gastrointestinal tract, because the contents were merely squeezed from it.

Balance. The soft-tissue balance, consisting of the thoracic organs and other tissues previously enumerated, contained less than 6% of the administered dose throughout the series, indicating that the values employed for total blood and total muscle were satisfactory, and that the separation of the soft-tissue ash from skeletal ash was relatively good.

Kidney. Initially the concentration of the light lanthanons in the kidney was about 1.6% per g, a much greater concentration than was found for any other soft tissue except liver. The kidney concentrated more of the heavy lanthanons than did any other soft tissue, about 0.9% per g. Eight months after administration the concentration in the kidney had, in all cases, decreased to low levels. There was no apparent correlation between either the 1-day or 8-month kidney concentrations and the quantity of the isotope excreted in the urine.

Excretion. The lanthanons are excreted both by the kidney and by the liver via the gastrointestinal tract. The relative importance of either excretion route for any given element of the series is dependent upon its position in the series. More than 50% of the administered dose of the lighter lanthanons is accumulated by the liver, and it is thereafter rapidly excreted in the feces. The heavier lanthanons are excreted primarily by the kidney. Those lanthanons in the middle of the series are excreted by both routes and to approximately the same extent. The cumulative excretion in both urine and feces is shown in Fig. 2 for Ce^{144} , $\text{Eu}^{152,154}$, and Tm^{170} . Very little of any of the lanthanons

† The tabular data are available from the authors upon request.

was excreted in the urine after the first week postinjection, although there was a slight tendency towards continued urinary excretion of the heavier members of the series. Most of the fecal excretion of Tm^{170} , and presumably of the other heavy lanthanons, occurred in the first two weeks after administration. Fecal excretion of the light and medium weight lanthanons occurred throughout the entire eight-month investigation, but at a slower rate after the first two weeks.

Liver. The major deposition site of the lighter members of the series is the liver. At the heavy end of the series the liver is a secondary deposition site and contains less than 5% of the initially administered radioisotopes. The retention of five representative members of the lanthanons in the liver is shown in Fig. 3. All the lanthanons are excreted from the liver, with half times ranging from 10 to 20 days, so that two months after injection less than 10% of that initially deposited remains.

Skeleton. The most important deposition site for all the members of this group is the skeleton. It is the primary target organ of the heavier lanthanons, and although initially it is apparently a secondary deposition site for the lighter members of the group, it becomes increasingly important because of their extended retention at this site. The skeletal retention of five representative lanthanons is shown in Fig. 4. The retention curves obtained for Ce^{144} and Pm^{147} (lighter lanthanons) show two components. One-third of these isotopes initially deposited in bone is somewhat labile with a half time of about fifteen days, and the remaining two-thirds is apparently firmly fixed. The curve obtained for $\text{Eu}^{152, 154}$ is unique in that it is convex. This presumably is due to the delayed deposition in the skeleton of $\text{Eu}^{152, 154}$, which was only gradually absorbed from the site of injection. The skeletal half time for $\text{Eu}^{152, 154}$, based on the best straight line through all the points is about 2.5 years. The retention curves for Tb^{160} and Tm^{170} were straight lines for which the half-time values are about 2.8 years.

Discussion. The data shown here establish

definite differences in excretion patterns and deposition sites between the light and heavy lanthanons; however these differences seem to be more a matter of degree than of kind. According to their behavior in the mammalian organism, the lanthanons can be divided into the same three groups familiar to modern lanthanon chemists: (3) the light lanthanons—lanthanum through samarium; the transition group—europium, gadolinium and terbium; and the heavy lanthanons—dysprosium through lutetium.

Radioautographs of long bones(5) and of costochondral junction(15) demonstrated that in the normal rat Ce^{144} and Pm^{147} (light lanthanons) are deposited on the endosteal and periosteal surfaces and in the vicinity of the small blood vessels in the compact bone. Copp *et al.*(16) compared the skeletal distribution in normal adult, normal young, and rachitic rats of the alkaline earths, Ce^{144} and Y^{88} . On the basis of chemical properties and metabolic behavior(16), Y^{88} can be grouped with the heavy lanthanons. They found that the skeletal deposition of Ce^{144} was moderately increased in normal young over normal adults, and strikingly increased in rachitic rats. The amount of Y^{88} deposited in the skeleton (about 70% of the administered dose) was unaffected by either rapid skeletal growth or phosphorous deficiency. Radioautographs of rachitic bone demonstrated that both Ce^{144} and Y^{88} were deposited almost entirely superficially in the shaft and in the uncalcified osteoid matrix below the epiphysis. All these findings strongly suggest that the lanthanons have a specific affinity for the bone protein even in the absence of bone salt.

The increased skeletal deposition of the heavier members of the lanthanon series and the difference in skeletal retention curves for the light and heavy lanthanons suggest that these two groups may be handled somewhat differently in the skeleton. It is likely that these differences are determined by the type or strength of the chemical combination of the lanthanons with the bone protein. Kettelle and Boyd(17) have made an interesting presentation of the effect of ionic radius on

the absorbability of the lanthanon ions on cation-exchange columns. They found that the sequence of relative absorbability was the exact reverse of the positions of these elements in the Periodic Table, *i.e.*, lanthanum was the most strongly absorbed and lutetium the least. This is in the same order as the decrease of basicity and increase in the stability of combinations with various chelating agents. It is this greater stability of the heavy lanthanon chelates that may be the basis for their greater skeletal deposition.

Summary. 1. The metabolism of the lanthanons has been studied in the rat on a systematic basis using tracer technics. High-specific activity preparations were administered orally or intramuscularly as citrate complexes. 2. Data are presented for the fifteen lanthanons 1 and 4 days after administration and at intervals up to 8 months for those isotopes with sufficiently long half lives. In general, absorption from the parenteral injection site was relatively complete at four days. Gastrointestinal absorption of the 4 lanthanons administered orally was insignificant. 3. The light lanthanons (lanthanum through samarium). Deposition was primarily in the liver and skeleton, 50 and 25% of the administered dose respectively. Elimination from the liver (presumably by way of the bile duct to the gastrointestinal tract) was quite rapid with a half-time of about 15 days. Two months after injection the skeleton retained about two-thirds of its initial deposition; there was no further elimination from the skeleton during the subsequent 8 months. 4. The transition lanthanons (europium and gadolinium). Deposition was more nearly equal in liver and skeleton, 30 and 40% of the administered dose respectively. Excretion was both fecal and urinary. 5. The heavy lanthanons (terbium through lutetium). Deposition was mainly skeletal, 50 to 65% of the administered dose. Elimination from the skeleton was slow with a half-time of approximately 2.5 years. Excretion of extra-skeletal heavy lanthanon occurred within the first two weeks after injection and was almost entirely urinary.

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Systemic Effects of 2-isovaleryl-1,3-indandione. (22176)

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(Introduced by J. H. Wills.)

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The 2-acyl-1, 3-indandiones possess anti-coagulant properties which make the more potent compounds useful as rodenticides(1-4). A typical acyl indandione, 2-isovaleryl-1, 3-indandione (Isoval) was found by Kabat *et al.*(2) to possess no significant effects on blood pressure, respiration, or plasma prothrombin time in cats after slow intravenous administration of 20 mg/kg for 2.5 hours. However, these authors noticed that after oral doses of 100-300 mg/kg given to rats and rabbits, labored respiration, progressive muscular weakness, hyperexcitability, pulmonary congestion, venous engorgement and systolic standstill resulted.

During our study of prolonged plasma coagulation time produced by Isoval in rabbits, we noticed that intravenous injection of comparatively large doses resulted in extremely rapid death (within 2 minutes). Because such an acute effect had not been reported previously it was decided to investigate this action of Isoval and related compounds.

Methods. Cats of mixed breed and sex were used. The animals were anesthetized with pentobarbital and tracheotomized for artificial respiration or for recording respiration by means of Stathan strain gauge attached to pneumotachograph. In some animals, respiratory recordings were made by means of an intrathoracic cannula. The right carotid artery was cannulated and connected to mercury U-tube manometer for blood pressure recording. All injections were made through a polyethylene tube cannula inserted into the right femoral vein. Spinal sections were performed through dorsal incisions at approximately the C-4 level. The sodium salt of Isoval is soluble in water to about 20 mg/cc. When solutions of greater concentration were required, the substance was dissolved in diethylene glycol monoethyl ether. The effect of epinephrine on the Isoval response was

determined by giving epinephrine in isotonic saline solutions before, together with, and after injection of the drug.

Results. Fig. 1 a and b show responses obtained at dose levels of 5, 20, 30, 40 and 60 mg/kg Isoval in single cats. The top tracing is respiration measured by means of intrathoracic cannula. As the dose of Isoval was increased, the hypotensive effect became more severe until at higher doses there was no recovery. Concomitant with the drop in blood pressure there was an increase in both rate of respiration and magnitude of intrathoracic pressure variations. Pneumotachographic recordings made on other animals showed that depth of respiration was also increased at higher dose levels.

Giving epinephrine together with and after the drug, had no appreciable effect on ultimate response to the drug. However, when 25 μ g/kg of epinephrine was followed by 60 mg/kg of Isoval, a milder response to the drug was seen. In one experiment 25 μ g/kg epinephrine was given after blood pressure had dropped to a low level (20 mm Hg) following injection of a mixture of 60 mg/kg of Isoval and 25 μ g/kg of epinephrine. No response to this second injection was seen immediately. However, after several minutes, a rapid increase of blood pressure to 100 mm of Hg was seen, followed by a later collapse with ultimate death.

On the assumption that some of the actions of Isoval were mediated centrally, a few experiments were done, in which the injections were given via the carotid artery. However, there seemed to be no change in pattern of blood pressure response from that obtained with intravenous injections.

Another approach, to determine any central effects, involved 3 different types of preparations: Cats with spinal cord sectioned at C-4; cats bilaterally vagotomized; and cats sectioned at C-4 and bilaterally vagotomized.

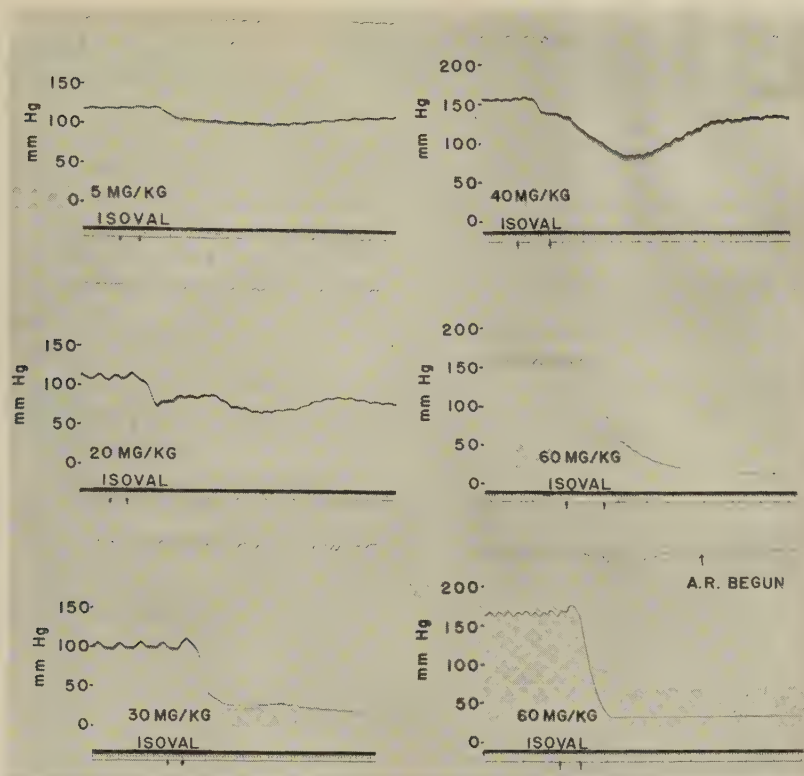


FIG. 1a (left). Blood pressure and intrathoracic respiration recordings at doses of 5, 20, and 30 mg/kg Isoval.

FIG. 1b (right). Blood pressure and intrathoracic respiration recordings at doses of 40 and 60 mg/kg Isoval.

In spinal vagotomized preparations, 2 animals responded to repeated doses of Isoval with only mild decrease in blood pressure and with subsequent recovery to preinjection levels. The first animal received 3 separate doses: 20, 20, 40 mg/kg; the second animals received 40, 40, 20 mg/kg. The first animal ultimately died.

Two similar preparations each received 40 mg/kg Isoval. There was no recovery from blood pressure reduction, which was as rapid as in intact animals.

In neither vagotomized preparations nor spinal sectioned preparations was there any difference in response to Isoval from that seen in intact animals.

Electrocardiographic recordings made of various animals showed no cardiac abnormalities to which the precipitous blood pressure fall could be attributed.

Discussion. When Isoval is administered intravenously to cats in adequate amounts, the principal effects obtained are: (1) rapid decline in blood pressure from which there is no recovery where the dose is large enough and, (2) a concomitant increase in rate and sometimes depth of respiration.

In high spinal-sectioned, vagotomized cats the blood pressure response was in 2 cases identical with that observed in intact animals. In 2 other cats, however, blood pressure decline appeared less drastic for a given dose than that observed in intact animals. Since there was variability even among intact animals in regard to dose required to produce death, it is difficult to determine if any of the spinal-vagotomized animals gave more or less response to a given dose. Vagotomized animals and spinal-sectioned animals with vagi intact did not differ from intact animals in

their response to the drug. From these observations it can be concluded tentatively that there is little if any centrally-mediated component in the drug response.

Electrocardiographic recordings showed no marked changes throughout the period of injection of the drug, at which time blood pressure was dropping rapidly. This does not rule out the possibility of a direct action of the drug on the heart. Murtha(5) in some experiments using papillary muscle preparations has been able to show some direct effects of Isoval on the myocardium.

Another possible explanation of the action of this drug is that sudden reduction in blood pressure may be due to rapid vasodilation in the vascular bed. Some slight evidence for this is seen in the fact that when epinephrine was given prior to injection of Isoval, a milder response was observed in some cases. This might imply that the vaso-constrictor action of epinephrine counteracts to some extent the vasodilation of Isoval.

It is interesting to note that Wakim *et al.* (6) used dicumarol and obtained results in dogs similar to ours. They attribute the action of acute doses of dicumarol to generalized vasodilation of the capillaries and small vessels, with a resulting vascular collapse. The fact that dicumarol and Isoval are both anticoagulants suggests that the mechanisms of their acute effects may be identical.

The experiments in which 2-pivalyl-1, 3-indandione (Pival) was used indicated a similar response to that obtained with Isoval. Pival seemed to have a somewhat greater hypotensive effect.

Preliminary experiments with 2-acetyl-1, 3-indandione, 2-phenylacetyl-1, 3-indandione,

2-diphenylacetyl-1, 3-indandione, and 2-benzoyl-1, 3-indandione indicated that these compounds exhibit similar effects. They were not investigated completely because of the difficulty of finding a suitable solvent for intravenous administration.

Conclusions. 1. 2-isovaleryl-1, 3-indandione when given intravenously to cats in doses ranging from 5-60 mg/kg produces a decline in blood pressure which increases in severity with increase in dose. 2. Concomitant with the decline in blood pressure there is an increase in respiratory rate and depth. 3. These effects cannot be attributed to direct action on the heart, nor do they appear to be centrally mediated. 4. It is suggested that rapid vaso-dilation, with consequent drop in blood pressure, may produce a fatal vascular collapse.

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Thiopental and Thyroid Metabolism.* (22177)

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Previous observations(1,2) indicated sodium ethyl (1-methyl-butyl)-thiobarbiturate (thiopental) to reduce the I^{131} uptake by the rat thyroid to 89%. Impairment of thyroid activity was not found related to the anesthetic effect, since anesthetics which did not contain a $N - \dot{C} = S$ grouping did not change the I^{131} uptake(1). Other anesthetic agents containing a thiocarbamate structure have also been shown to have anti-thyroid activity (3). Widespread use of thiopental as an anesthetic agent suggested that its antithyroid properties be further investigated. This study indicates a relationship between metabolism of thiopental by thyroid and ability of thyroid to accumulate I^{131} .

Methods. The rats used in these studies were of the Sprague-Dawley strain, males, 180-200 g. Hypophysectomized rats were all males, average weight 155 g, and were used 26-30 days post operation. The basal ration consisted of commercial grains and water *ad libitum*. Hypophysectomized animals were given a daily supplement of oranges and lettuce. Not less than 8 nor more than 10 animals were used in each experimental group. The I^{131} uptake and assay procedures have been described previously(1,3). Total iodine content of thyroids was determined by modification of the method of McClendon and Foster(4). S^{35} labeled thiopental was obtained from Abbott Laboratories. It was administered in 10 μ c doses diluted with non-radioactive thiopental to give animals a 40 mg/kg dose as used in earlier experiments. Analysis of S^{35} in tissues was achieved following oxidation (HNO_3 and $HClO_4$) of tissue sulfur to sulfate which was precipitated with benzidine hydrochloride and assayed in a gas-flow detector. Sulfur content of precipitate was determined by titration of benzidine sulfate with 0.01 N NaOH. Counting was extended to a 2% statistical error. Corrections for decay

and self-absorption were applied by conventional methods. Results are expressed as specific activity, defined here as counts per minute (CPM) per μ M. In some preliminary experiments, results were expressed as CPM/mg tissue. In the experiment wherein both I^{131} and S^{35} were used in the same animal, the I^{131} was assayed in the thyroids first using a thick-window (3.5 mg/cm^2) G.M. counter which was insensitive to soft beta radiation of S^{35} as determined by previous trial runs with similar amounts of labelled thiopental. Iodine content was determined on pooled single lobes of thyroid while sulfate content was determined on the remaining pooled lobes. In some cases, it was necessary to add known amounts of carrier to effect chemical separation of the sulfate.

Results. Thiopental inhibited thyroid activity immediately following administration (Fig. 1). Response of thyroid in a 4-hour test period was inversely related to the amount of thiopental given to the animal (Table I). Of perhaps even more interest was the observa-

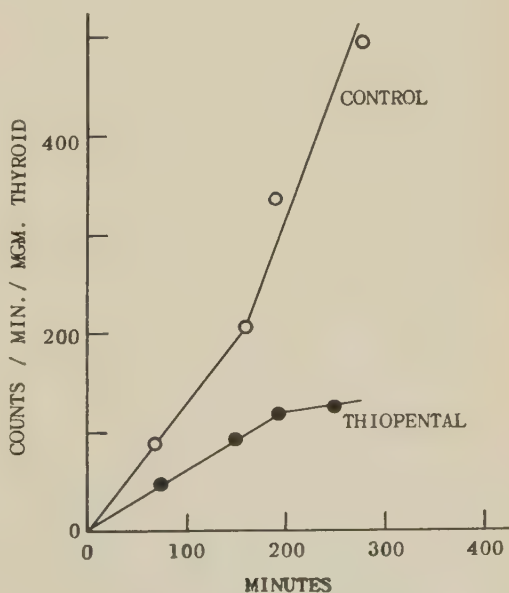


FIG. 1. Effect of thiopental (40 mg/kg) on I^{131} incorporation into rat thyroid; 5 μ c I^{131} .

* These studies were supported in part by a grant from the Office of Naval Research and the English Fund of the Hahnemann Medical College.

TABLE I. Four-Hour Uptake of I^{131} by Rat Thyroid in Relation to Amount of Thiopental Administered. $2\ \mu C\ I^{131}$ intraperitoneally.

Dose (mg/kg)	I^{131} CPM/mg	%
0	$214 \pm 19^*$	100
5	138 ± 15	64.4
10	112 ± 14	52.3
20	76 ± 9	35.5
40	44 ± 7	20.6
70	26 ± 5	12.2

* Stand. error of the mean.

tion of impairment of thyroid activity following administration of a single 40 mg/kg dose of thiopental (Table II). It required 6 to 7

TABLE II. Recovery of I^{131} Trapping Ability of Thyroid following a Single Dose of Thiopental (40 mg/kg).

Hr	I^{131} uptake (% controls)
4	18
12	33
24	47
48	56
72	62
96	64
168	96
192	100

days for complete recovery. To obtain information on the nature of the inhibition, I^{131} uptakes were performed on hypophysectomized animals. The results shown in Table III indicate that thiopental reduces the uptake

TABLE III. Effect of Thiopental on I^{131} Uptake by Thyroids of Hypophysectomized and Normal Rats.

Group	CPM/mg	%
Hypox	$28.8 \pm 5.7^*$	100
Hypox + thiopental	6.8 ± 0.7	23.6
Normal	258.0 ± 25.4	100
Normal + thiopental	30.1 ± 4.7	11.6

* Stand. error of the mean.

of I^{131} in the absence of the pituitary and suggest that the principal mode of action is via interference with iodine uptake, probably by reduction of I_2 to I^- (5). In another experiment, normal animals were given I^{131} and samples were taken for thyroid assay at 24 hours. Remaining groups were given thiopental (40 mg/kg) and thyroids assayed (1-3) 24 hours later. Compared to controls the treated animals lost I^{131} rapidly (Fig. 2). A final experiment was performed wherein S^{35}

labelled thiopental was administered along with carrier-free I^{131} to intact and hypophysectomized rats. The results indicated that recovery of the thyroid was related to disappearance of S^{35} from thiopental in the thyroid (Fig. 3). The S^{35} content of pituitary and adrenal seemed to bear no relation to thyroid iodine metabolism. Specific activities of the normal rat adrenal S^{35} at 2, 24, 48, 120, and 144 hours post thiopental were 93.5, 7.9, 2.4, 2.0, and 1.8; of the pituitary 4.1, 2.6, 1.9, 2.2. The levels of S^{35} from thiopental in the endocrines of the hypophysectomized rats were lower than controls and a constant level of S^{35} in the thyroid of hypophysectomized rats appeared to be related to a constant I^{131} uptake (Fig. 3). The specific activities of the hypophysectomized rat adrenal S^{35} were 4.4, 2.0, and 1.8 at 24, 48, and 120 hours.

Discussion. The data presented here suggest that thiopental possesses properties of an antithyroid substance predictable by its thiocarbamate structure. That it also possesses anesthetic properties is indeed most interesting. Prolonged inhibition of thyroid activity demonstrated by a single dose is of interest in view of the observations of others (6,7) that thiopental is rapidly removed from the blood stream and stored in body fat from which it is slowly released. Further, studies on metabolism of thiopental (7,8) indicate it to be diversely attacked by the organism, thiourea being one of the many metabolic products. It may be that thiourea or substituted thioureas formed by metabolism of thiopental may be ultimately responsible for the prolonged in-

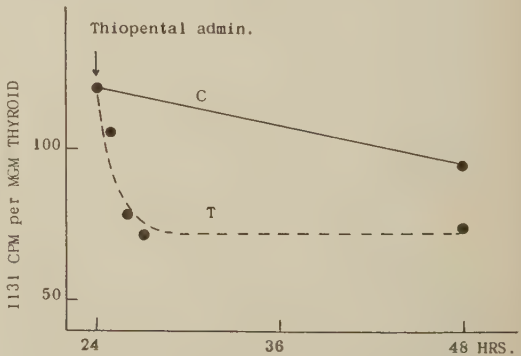


FIG. 2. Reduction of I^{131} content by administration of thiopental (40 mg/kg) to rats given $1\ \mu C\ I^{131}$ 24 hr previously.

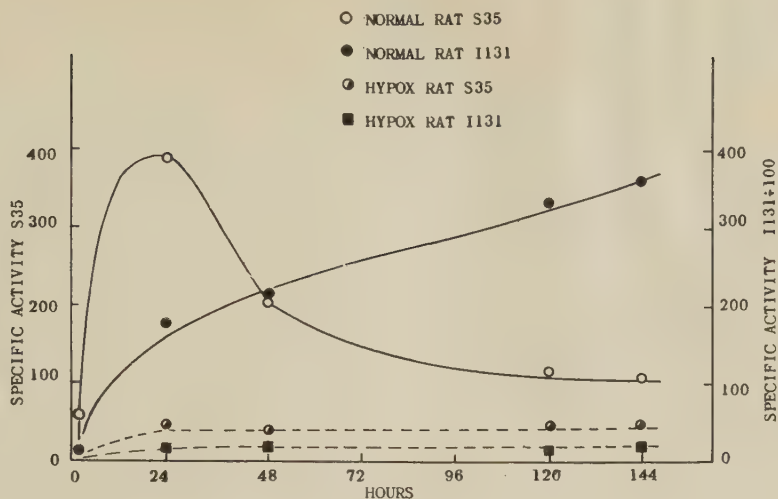


FIG. 3. Thyroid I^{131} in relation to S^{35} from thiopental in normal and hypophysectomized rats.

hibition. The relatively long period wherein activity from the S^{35} thiopental was found in the thyroid (Fig. 3) supports this hypothesis. The S^{35} activities of the pituitary and adrenal show no obvious relation to inhibition of the thyroid suggesting that other endocrines are not involved. The S^{35} and I^{131} specific activities of thyroids of hypophysectomized rats were both low and did not change during the experiment. Since thyroid in these animals was not functioning properly due to absence of the thyrotrophic hormone, these results were not unexpected.

In vitro studies by investigators indicated that aqueous thiopental solutions reduced I_2 to I^- . The reduction was not stoichiometric, more than molar equivalents of I_2 were reduced by thiopental. These observations suggest that the reaction is quite complex. This phenomenon is characteristic of many anti-thyroid agents(9). It would appear that thiopental could deprive the thyroid of I_2 by reducing it to I^- in the organism. This view is supported further by the reduced uptake of I^{131} in the hypophysectomized rat treated with thiopental. The ratio of inorganic I^{131} to diiodotyrosine I^{131} to thyroxine I^{131} was 54; 33; 13, and 57; 28; 15 in treated and non-treated animals respectively (4-hour uptake). These nearly identical ratios of activities indicated that thiopental did not interfere with thyroxine synthesis but functioned by depriving

the thyroid of iodine. Plasma PBI^{131} in thiopental-treated rats was also reduced to 49% of control values, another indication that the primary action is a reduction of available I_2 for synthetic purposes.

Summary. 1. Thiopental influences thyroid metabolism primarily by depriving the thyroid of available I_2 . 2. The prolonged inhibition of thyroid activity by thiopental is probably related to accumulation of its metabolic products in the thyroid.

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Some Properties of Human Serum Lipoproteins.* (22178)

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Our concern with the lipides of serum has been principally with respect to their characterization as lipoproteins. In this regard we have been able to demonstrate, by a variety of technics, that carotene, acetal phosphatidyl choline and probably sphingomyelin are characteristic of human β_1 -lipoprotein(1,2); carotene had already been shown by others (3) to be associated with β_1 -lipoprotein. The presence of acetal lipides in serum is not a new observation(4); only recently, however, have studies directed toward demonstrating the protein association of serum acetal phosphatides been reported(1,5). We have been unable to demonstrate a lipide component characteristic of α_1 -lipoprotein but do find vit. A to be regularly associated with albumin.

Methods. Preparative Ultracentrifugation. 200 to 400 ml of blood were obtained by veni-puncture from normal, postabsorptive, human subjects and let clot undisturbed at 20°C for an hour. The serum was separated by centrifugation in an International refrigerated centrifuge (15°) and promptly submitted to ultracentrifugation for 8-10 hours at 4-12° using a Spinco E5 rotor at 52640 rpm (ca 200000 g). Appearance of the tubes after centrifugation is diagrammed in Fig. 1. Fractions 1-5 were quantitatively collected by means of a specially constructed aspirating device and examined by one or more of the following procedures: 1) Refractionation in the ultracentrifuge after adjusting density to (a) 1.063 with NaCl or to (b) 1.21 with NaCl and KBr or with D₂O and NaCl. Four to 6 hours at 200000 g with the E5 rotor sufficed to float the desired fraction (β_1 -lipoprotein at 1.063 and both α_1 and β_1 -lipoproteins at 1.21) into the top ml which

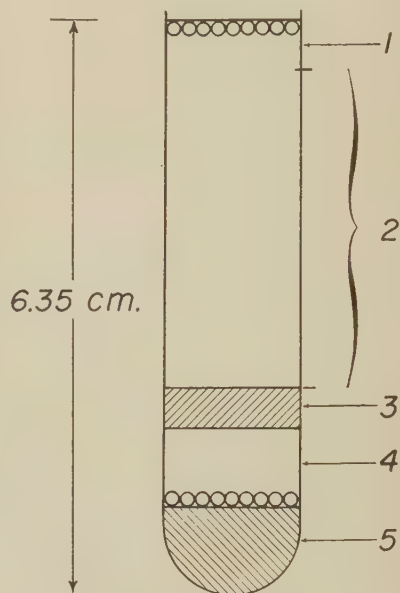


FIG. 1. Serum following 8 hr ultracentrifugation; (1) water-clear to milky; (2) water-clear; (3) bright orange-yellow, turbid; (4) yellow, clear; (5) yellow-brown, clear and includes thin flocculent layer dividing 4 from 5. Typical volume recoveries are 0.6, 3.6, 0.5, 0.7, and 1.0 ml respectively.

was collected by aspiration. 2) Analytical schlieren analyses of all samples were performed after adjusting the density to 1.063 or to 1.21. For these analyses the Spinco An-A rotor operating at 52640 rpm was employed, utilizing both visual inspection of the viewing screen and photographs in the analysis. 3) Chemical analysis on (a) chloroform:methanol (4/1:v/v) extracts and (b) benzene extracts of frozen-dried material. Phosphorus and choline were estimated as described earlier(6); cholesterol(7); total lipide esters (8); carotene from its absorption spectrum (9); and vit. A using both the Carr-Price reaction(9) and the technic of Bessey, *et al.* (10). As here measured choline was an estimate of the lecithin (phosphatidyl choline) and of acetal phosphatidyl choline; sphingomyelin may be estimated by difference from

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TABLE I. Typical Data from Chloroform: Methanol and Benzene (in Parenthesis) Extracts of Frozen-Dried Serum Fractions Obtained by Ultracentrifugation as Described in the Text.

Frac- tion	Color of extract*	Phosphorus		KOH hy- drolyzable choline 10 ⁻⁴ mM/ml fraction	Cholesterol		Total carboxylic esters	Carotene	Vit. A	Acetal lipide
		Total	KOH hy- drolyzable		Total	Non- esterified				
1	W (W)	14.4 (6.3)	12.3	0	10.0 (10.0)	5.1 (5.0)	198 (163)	0 (0)	0 (0)	0 (0)
2	W (W)	4.4 (0)	4.3	0	3.1 (3.0)	2.0 (2.0)	63 (38)	0 (0)	0 (0)	0 (0)
3	Y (Y)	150.0 (4.8)	113.2	85.0	253.1 (171.3)	56.2 (55.7)	735 (200)	4+ (4+)	1+ (0)	4+ (±)
4	Y (W)	64.0 (0)	55.1	38.8	54.4 (1.3)	9.0 (1.1)	270 (0)	± (0)	2+ (0)	+ (±)
5	Y (W)	38.5 (0)	33.8	19.4	28.3 (1.0)	3.1 (0.5)	145 (0)	0 (0)	4+ (0)	± (±)

* W = White; Y = Yellow.

the total and KOH phosphorus values(6). The desirability of using frozen-dried preparations in a study of this kind has been elaborated elsewhere(11). 4) Paper chromatographic analysis of both chloroform: methanol and benzene extracts(12). 5) Paper electrophoresis (ionophoresis) both a Kunkel-type apparatus and a Spinco Model R were used at pH 8.6 (0.05 M sodium barbital) usually operating at 250 volts, 2 m.a. for 20 hours. Whole serum was submitted to the techniques described above to aid in interpretation of the fraction data.

Results. With respect to their lipoprotein content, fractions 1 and 2 were normally quantitatively insignificant, together accounting for only 7% of the serum cholesterol and 14% of the phosphatide although occupying 66% of the volume. Fraction 1 consisted of chylomicrons which were principally triglyceride and cholesterol(2), all of which was readily extractable from frozen-dried samples by benzene, while only 50% of their phosphatide was so extracted (Table I). Fraction 2 was characterized by its low lipide content; it should be recognized that this fraction is adjacent, above and below, to fractions of high lipide content (Fig. 1).

Fraction 3 usually contained all of the β_1 -lipoprotein and perhaps 50% of the α_1 -lipoprotein as judged by the schlieren tracings (Fig. 2). The β_1 -lipoprotein was easily sep-

arable from α_1 -lipoprotein by ultracentrifugation at density 1.21. This separation was accomplished in the An-A rotor; centrifugation continued until, by inspection of the viewing screen, the yellow β_1 -lipoprotein peak disappeared to the left; the α_1 -peak was then nearly halfway across the field. Removal of the analytical cell from the rotor permitted aspiration of the subnatant solution which

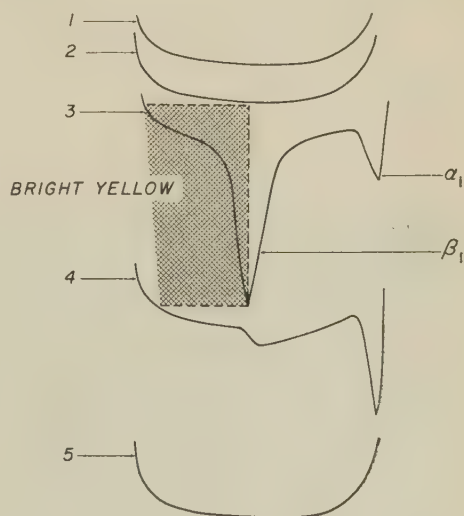


FIG. 2. Flotation schlieren patterns (22 min. pictures) from fractions 1-5 after adjusting density to 1.21 with D₂O-NaCl. Occasionally an α_1 -peak was observed in 5. When adjusted to density 1.063 the β_1 -peak had an S_r of 6. The yellow color of the β_1 -peak in curve 3 was readily observed on the viewing screen during the run.

TABLE II. Representative Cholesterol-Phosphatide, Free-Total Cholesterol Mole-Ratios of Various Human Blood Lipoprotein Fractions: (a) This Paper, (b) Others.

Source	Cholesterol	Free cholesterol
	Phosphatide	Total cholesterol
a. Fraction 1	.69	.51
2	.68	.51
3	1.9	.27
4	1.0	.20
5	.75	.14
D ₂ O • NaCl β_1	2.8	.28
α_1 *	1.6	.25
Diabetic β_1 †	1.8	.49
b. Cohn fractionation(3)		
β_1	2.1	.27
α_1	1.4	.27
Starch ionophoresis (13)		
β_1	2.1	.27
α_1	.58	.22
Ultracentrifugation(3)		
β_1 (S ₁ 4)	2.4	.25

* Shown not to be free of β_1 .

† Obtained by preparative ultracentrifugation of serum from patient in diabetic acidosis. This material collected at the top of the tube as an orange-yellow waxy layer similar to Hunter's(2) cream-layer β_1 .

contained the α_1 -lipoprotein and some β_1 . The bulk of the β_1 -lipoprotein remained on the upper face of the cell as an orange wax, easily lifted off; these materials were then examined by ionophoresis, paper chromatography, etc. (Table II). As in the case of ionophoresis of fraction 3, these β_1 samples

migrated more rapidly than the β_1 -lipoprotein in whole serum (Fig. 3), probably due to the absence of *plain* β_1 -globulin. In all cases β_1 -lipoprotein was characterized by intense osmiophilia, evidently due to the carotene, and by a strong reaction for acetal lipid. By means of various spot-tests and paper chromatography(12) the major acetal lipid component of β_1 -lipoprotein was identified as acetal phosphatidyl choline(2,12). The α_1 -lipoprotein was identified through its paper electrophoretic mobility on staining with bromophenol blue; it was not osmiophilic and did not stain for acetal lipid. Albumin and non-lipide globulins were absent from the D₂O • NaCl preparations.

Fraction 4 usually contained the rest of the α_1 -lipoprotein as demonstrated by ionophoresis and by ultracentrifugation at density 1.21 in D₂O • NaCl. Similarly fraction 5 was shown to contain most of the vitamin A (osmiophilia in the albumin region, *cf.* Fig. 3) and occasionally some α_1 -lipoprotein. In the D₂O • NaCl refractionation the vitamin A was found to be below the top milliliter.

Ionophoretically, fractions 1 and 2 were negative except for traces of albumin. Fraction 3 showed a trace of γ -globulin, all the β_1 -lipoprotein, some α_1 -globulin (lipoprotein?) and a moderate amount of albumin. Fraction 4 showed a moderate amount of γ -globulin, no

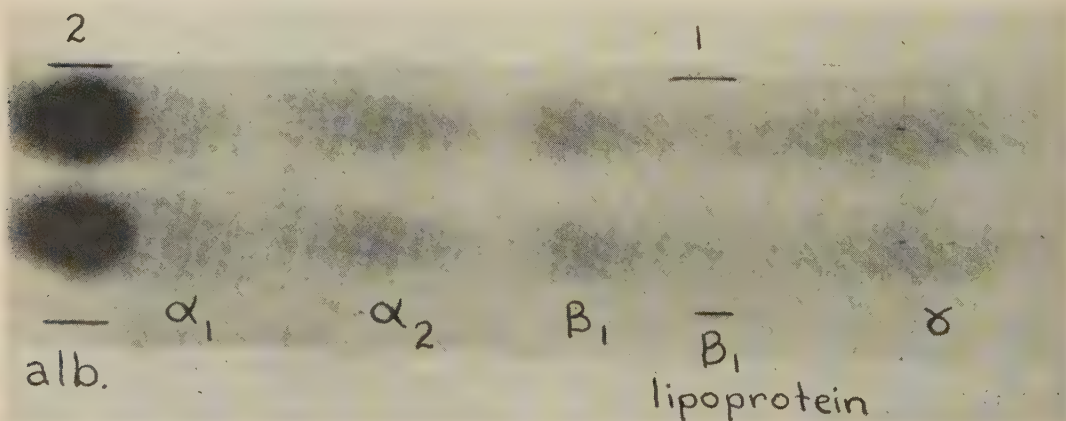


FIG. 3. Ionophoresis of 10 lambda aliquots of 2 normal human sera. The paper was dried promptly at room temperature by means of a fan and exposed to OsO₄ vapors for 15 min.(12) resulting in a browning in the zones delineated by the pencil lines 1 and 2. The paper was then stained for protein using bromophenol blue. On duplicate specimens it was demonstrated that usually only the β_1 osmiophilic region was positive for acetal lipid.

β_1 -lipoprotein, considerable β_1 , α_2 and α_1 globulins and albumin. Fraction 5 was similar to 4 except for markedly diminished α_1 and a marked increase in the other globulins and albumin.

Discussion. The behavior of frozen-dried β_1 -lipoprotein in chloroform: methanol and in benzene as here described (Table I) renders less useful the notion that all protein-bound lipides can be estimated by the solvent partition method(6,12). In this case carotene and cholesterol, for example, seem to be loosely bound, *i.e.*, extractable by benzene. On the other hand it is apparent that the other blood lipoproteins (excepting chylomicrons) are more resistant to benzene extraction.

Summary. Normal, postabsorptive, human serum was fractionated by means of the ultracentrifuge and the lipoproteins studied with the aid of paper chromatography, ionophoresis and by chemical procedures. The characteristic features of β_1 -lipoprotein and methods for its isolation are described.

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Development of Arthritis, Periarthritis and Periostitis in Rats Given Adjuvants.* (22179)

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In this investigation skeletal muscle was incorporated into a Freund-type water-in-oil emulsion and the mixture administered by various routes to rats. Within a short time, ranging from 14 to 45 days, a high percentage of the animals which received intracutaneous inoculations began to develop tender swellings of the ankles, the paws, the tail and the digits. Single or multiple joints were involved. The experiment was repeated on two further occasions. In the third trial a different strain

of rats was used. The original results were readily reproducible.

Materials and methods. Originally both male and female rats of the Wistar strain were used. They ranged in weight from 230 to 280 g. The final series contained female rats of the Long-Evans strain, weighing 180 to 220 g. All animals were fed a standard pellet diet of Purina chow and water *ad libitum*. They were weighed frequently and inspected almost daily throughout the investigation period. The inoculum was prepared as follows: a healthy rat of the same species to be subsequently used in the study was killed

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TABLE I. Incidence of Clinical Alteration of Extremities and Tail in Relation to the Site of Inoculation—Series 55A1.

Site of inoculation	No. of rats (Wistar)	Severity of change				% animals affected
		None	Slight	Moderate	Severe	
Intracutaneous (posterior cervical)	17	4	6	2	5	77
Subcutaneous	8	6	1	1	0	25
Intraperitoneal*	4	0	0	0	0	0
Controls (no inoculations)	8	8	0	0	0	0

* Produced fibroplastic peritonitis but no peripheral alteration.

Mean time of alteration after initial injection—23 days (15-32 days). Avg No. of injections before alterations—2.9 (1-5).

by a sharp blow. Skeletal muscles from the thigh and the iliopsoas groups were freed from as much surrounding fat and connective tissue as possible and weighed in a sterile beaker. Ten grams of this muscle was homogenized in a Waring micro-blendor with 30 ml of sterile isotonic saline solution using intermittent action of the rotor blades to avoid overheating of the mixture. The resultant homogenate was then compressed through fine-mesh sterile gauze and to the filtrate was added 32 ml of refined thin mineral oil, 8 ml of the emulsifier Falba,* and 100 mg of heat-killed *Myco. phlei*. The mixture was emulsified by the syringe method in a sealed vial and stored at 4°C. It was warmed to 50°C for a few minutes each time inoculations were given. In the final series aqueous Merthiolate, to a final dilution of 1:10000, was added without significant effect on the final result. In one portion of the final experiment muscle tissue was excluded from the inoculum and was replaced by an approximately equal amount of sterile isotonic saline solution. In another portion of this experiment only saline and muscle were combined so that the final concentration of muscle homogenate was approximately the same as in the mixture with adjuvants added. All animals were given 0.1 ml of the emulsion at each inoculation. Injections were given in a series of 3 at 2-day intervals, then a 14-day rest period was followed by a second and in some animals a third and fourth series of injections. In the first experiment inoculations were given intracutaneously into the skin of the posterior cervical region after first carefully clipping the

hair. Subcutaneous injections were also given into this region. A small group of animals were given intraperitoneal injections. In the second and third experiments all inoculations were given intracutaneously. A 21 gauge needle and tuberculin syringe were used for all injections.

Results. A total of 97 animals were used in the study which was divided into 3 experiments. The results of each will be presented separately but the discussion of the clinical and pathological changes will be combined since they were essentially similar in all groups. A detailed autopsy was performed on every animal that has been sacrificed. Some animals showing clinical change have been allowed to recover and are under continued observation.

Controls. Unrelated Deaths. Uninjected control animals from the same litter groups as the treated animals have been followed in each experiment. Clinically and at autopsy these have consistently failed to demonstrate any lesions of the joints. Two animals in the treated and one in the control group died from acute pneumonitis.

First Exp. Wistar rats were given inoculations by various routes. The results are shown in Table I and demonstrate the superiority of the intracutaneous mode of injection. The incidence and degree of response was similar in both sexes. *Second Exp.* The initial experiment was repeated, again with Wistar rats and a freshly prepared inoculum. All injections were given intracutaneously and the initial results were essentially reproducible as shown in Table II. *Third Exp.* Long-Evans female rats were used and the content of the inoculum was varied to determine the

† Obtained from the McNerney Chemical Corp., Los Angeles, Calif.

TABLE II. Incidence of Clinical Alteration of Extremities and Tail—Intracutaneous Inoculations.

Series	No. of rats (Wistar)	Severity of change				% animals affected
		None	Slight	Moderate	Severe	
55A 13-15	16	6	4	4	2	62
Controls	8	8	0	0	0	0

Mean time of alteration after initial injection—28 days (20-45 days). Avg No. of injections before alteration—4.1 (3-6).

relative value of various components in inciting the pathologic changes as noted. The results show that although muscle tissue does not appear to be necessary for the development of these changes it may have a potentiating effect. Acid fast bacilli and probably the mineral oil are essential. The incidence of clinical alteration in this experiment is shown in Table III.

Time of Onset and Clinical Appearance of Limb and Tail Alterations. The earliest alterations occurred on the fourteenth day after the original inoculation. Initial alterations in other animals were delayed up to 45 days. The average time of onset was 22 days. The first changes usually were seen in the ankles, hind paws or proximal or middle phalangeal joints of the feet. In general the earlier the onset the more severe the clinical involvement but this was not always the case. In a small series, not included above, only one intracutaneous inoculation was given and 3 out of 8 animals have shown joint alterations. The ankles and tarsal regions were most frequently affected severely; then the forepaws, digits and tail. When markedly affected, serum exuded from the swollen red paws which were obviously painful and tender to touch. Frequently one or several digits would be completely spared from swell-

ing although adjacent ones were 2 or 3 times their normal size (Fig. 1). The tail was more frequently involved in the Wistar strain and showed swelling, nodulation and desquamation, more marked in the proximal segments. Many severely affected animals lost weight. Lesser degrees of swelling affected many other animals and again the ankles were most frequently involved. Fusiform red swelling of one, several or all of the proximal interphalangeal joints was not uncommon (Fig. 2) especially in the Wistar strain. Diffuse digital swelling was more commonly seen in the Long-Evans strain. In animals that were observed for several weeks or months there was a gradual return of lost weight as evidence of acute inflammation subsided. Functional recovery did not continue beyond 3 months at which time there were still many residuals in the form of chronic firm enlargements especially about the ankle joints (Fig. 4), the metatarso- and metacarpophalangeal joints which showed typical flexion deformities (Fig. 3) resembling a "flipper hand," and large rigid tails. Firm ankyloses often partially or almost completely restricted motion especially at the ankles and in the tail joints. Histological study has not as yet been made on these chronic alterations. *Roentgenologic Observations.* After one month, early bone

TABLE III. Incidence of Clinical Alteration of Extremities and Tail with Varying Composition of Inoculation Material—Series 55A16-21.

Inoculum content	No. of rats (Long-Evans)	Severity of change				% animals affected
		None	Slight	Moderate	Severe	
Adjuvant + muscle + merthiolate (1:10,000)	8	3	2	1	2	62
Adjuvant alone*	8	5	2	0	1	37
Adjuvant (no acid fast bacilli) + muscle†	8	8	0	0	0	0
Saline + muscle	6	6	0	0	0	0
Controls (no inoculations)	6	6	0	0	0	0

* Small granulomas at injection site.

† No granulomas at injection site.

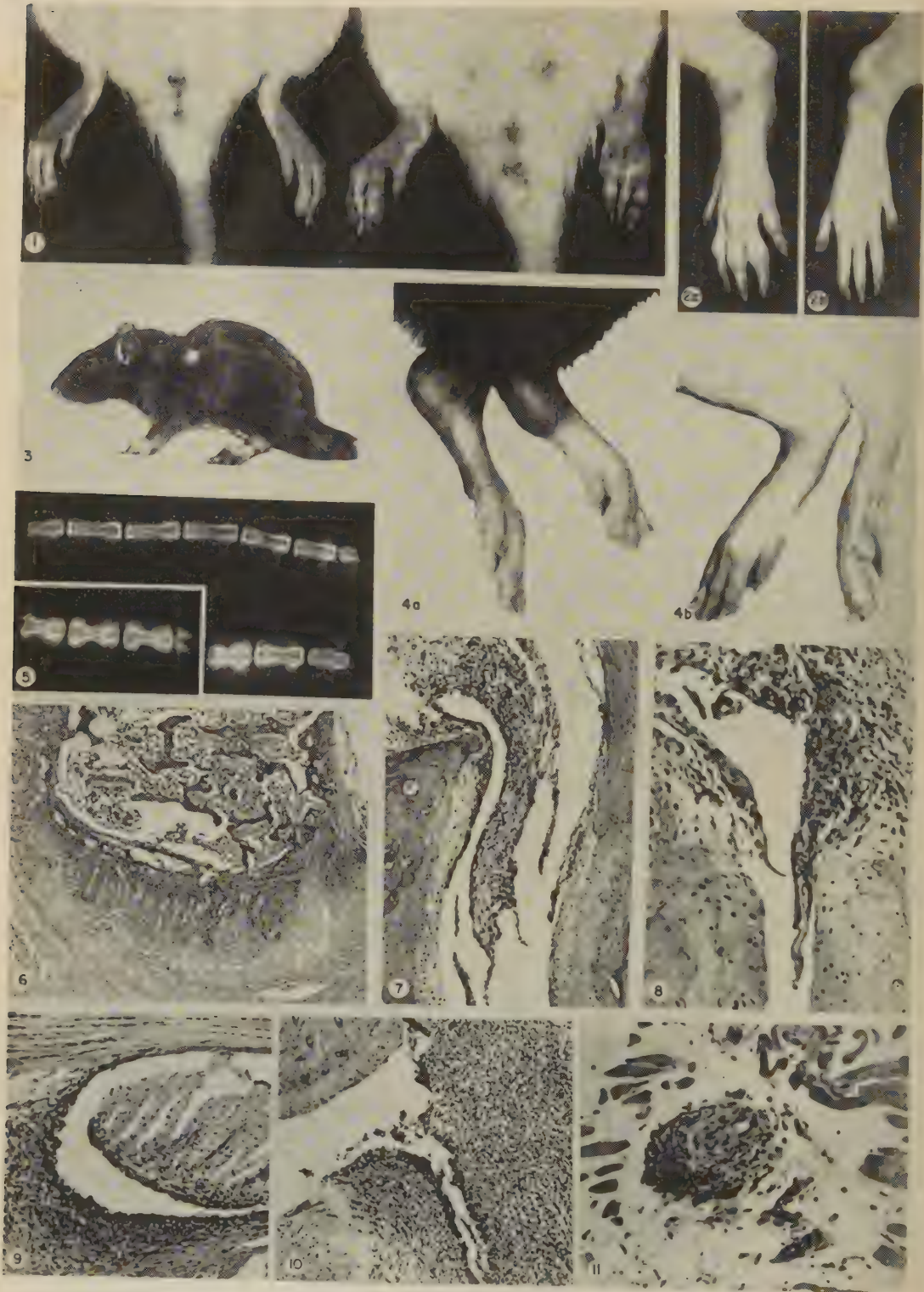


FIG. 1. Diffuse swelling of both tarsal regions and digits compared with control animal. Note absence of involvement of middle toes.

FIG. 2. (a) Fusiform swelling of proximal interphalangeal joints. (b) Control.

FIG. 3. Flexion deformities at metacarpo- and metatarsophalangeal joints. Duration of disease, 4 mo. Retouched photograph.

FIG. 4. (a) Chronic firm swelling of ankles with marked restriction of ankle motion. (b) Control.

FIG. 5. X-rays of tail vertebrae. The upper picture is a control. Lower X-ray demonstrates increased periosteal density, exostoses and narrowing of intervertebral disc spaces.

FIG. 6. Section through os calcis showing active periosteal osteogenesis and inflammatory reaction on the right with invasion by granulation tissue through the bony cortex and into the marrow cavities in this area. $\times 45$ (before reduction).

FIG. 7. An ankle joint showing a broad, edematous, inflamed synovial villus projecting into the joint space. Note extension of granulation tissue over the surfaces of the articular cartilage. $\times 180$ (before reduction).

FIG. 8. Fibrinoid necrosis of synovial margin of a tarsal joint. $\times 220$ (before reduction).

FIG. 9. Peritendinitis and tendonitis. $\times 130$ (before reduction).

FIG. 10. Intense inflammatory reaction, granulation tissue growth and synovial reduplication at the margin of a phalangeal joint. Note neutrophils in joint space. $\times 180$ (before reduction).

FIG. 11. Perivascular lymphocytic collection about a small intramuscular artery in one of the small muscles of the foot. $\times 220$ (before reduction).

and joint changes were visualized. These were progressive during the "healing" phase and were maximal at 3 months. They were most marked about the os calcis and the ankle joint but were often intense on the lateral wings of the caudal vertebrae (Fig. 5). The change consisted essentially of a periostitis followed by the appearance of nodular exostoses along the entire surface or an isolated segment of bone. The joint spaces of the ankle were narrowed or completely obliterated and the intervertebral spaces of the tail were narrowed. Oblique views of formalin-fixed spine showed some bony reaction with partial or complete obliteration of the apophyseal joint spaces in the lower dorsal and lumbar spine. No other joints were altered radiographically.

Histopathologic Observations. Animals were sacrificed at various stages during the development and subsidence of the clinically observed bone and joint lesions. The process was essentially one of intense stimulation of the connective tissue elements with focal and diffuse inflammatory infiltrations. This response was limited to the regions about the affected bones, joints and tendons and rarely extended into the subcutaneous tissue. The overlying dermis was normal. Edema, fibroblastic proliferation and sparse mononuclear or polymorphonuclear infiltrations were noted in the earliest stages. After 10 or more days there was exuberant proliferation of granulation tissue about some bones, joints, tendons,

synovial sheaths and synovia of the joints. This tissue was responsible for the focal swellings and hyperemia seen clinically. It was infiltrated to a variable degree, often intensely, with neutrophils, plasma cells, lymphocytes and mononuclear histiocytes. Eosinophiles were rarely seen. The next most striking observation was the profound osteoblastic activity which arose mainly from the periosteum of cortical bone and rapidly formed broad spicules of new bone (Fig. 6). This bone was frequently surrounded at its outer border by a zone of immature cartilage which appeared to arise *in situ* in the adjacent hyperplastic connective tissue. Granulation tissue frequently invaded the cortical bone and spread out into the marrow cavity (Fig. 6). The synovia about tendon sheaths and joint spaces was hyperplastic and portions of tendon were often invaded (Fig. 9) and adherent to the side of the sheath. The synovial villi were enlarged, edematous and inflamed and frequently projected deeply into the joint spaces (Fig. 7). Occasionally fibrillar material surrounded the joint margin and resembled fibrinoid necrosis (Fig. 8). Small numbers of neutrophils were present in some joint spaces (Fig. 10) along with an apparent slight increase in joint fluid. There was no primary involvement of the articular cartilages but granulation tissue from the margins of the synovial membrane was frequently observed growing over the surfaces of the articular cartilages (Fig. 7). The

above changes were seen in the ankle joints or the joints of the paws or fingers when clinical involvement was apparent. Lesser degrees of connective tissue reaction were occasionally noted about bones and joints not grossly affected.

Sections of the tail showed an essentially similar process plus early invasion of the margins of the intervertebral discs and the subchondral spaces by inflamed granulation tissue. Other findings of interest were focal lymphocytic and mononuclear cellular infiltrates in skeletal muscles near involved bones and joints. This was a true focal polymyositis with destruction of one or several striated muscle fibers. An arteritis or vasculitis was infrequently noted in the regions of bone and joint involvement (Fig. 11). It consisted of lymphocytic and mononuclear cellular infiltrates about a portion or all of a small intramuscular artery. There was no necrosis of the vessel walls. Focal areas of edema and necrosis, without surrounding palisading, were occasionally seen in the hyperplastic connective tissue. Occasional focal subcutaneous lymphocytic collections were noted.

Similar histologic changes were not noted in muscle and other tissues remote from the areas of clinical involvement. All other organs were histologically negative except for an occasional small granulomatous focus in the liver and varying degrees of a granulomatous pneumonitis in some animals. Similar pulmonary lesions have been frequently noted in guinea pigs following repeated injections of a Freund-type adjuvant(1). In summary the pathological changes consisted of: connective tissue proliferation and granulation tissue formation; acute and chronic inflammatory infiltrates; periostitis with periosteal and enchondral new bone formation; arthritis and peri arthritis; synovitis; tendonitis and peritendonitis; focal myositis (near affected bones and joints only); perivascularitis, non-necrotising (rare).

Discussion. There have been many previous attempts to produce an arthritis in animals. Some of these have been successful by such varied methods as injecting large amounts of desoxycorticosterone acetate into

rats previously nephrectomized and adrenal-ectomized(2), inoculating rabbits repeatedly with large amounts of horse serum(3) or killed beta hemolytic streptococci plus chondroitin sulfate(4), and by injection of viable pleuropneumonia-like micro-organisms into mice and rats(5).

The pathogenesis of the bone, joint and connective tissue alterations reported herein must at present remain a matter of speculation. There is suggestive evidence that hypersensitivity is an important factor in these reactions when it is considered that a known allergenic emulsion was used as the inciting agent. Studies are underway to determine if activation of pleuropneumonia-like micro-organisms are responsible for these reactions. Periosteal osteogenesis has not been recorded as a feature of this infection, however it is a prominent factor in experimental lathyrism (6).

Pathologically the basic lesion appears to be an intensive focalized mesenchymal tissue response. There are several gross and microscopic similarities between these lesions and those observed in human rheumatoid arthritis but since it is likely that such tissues can only react in a limited number of ways to many stimuli the identification of such changes with any human counterpart is unwarranted.

Summary and Conclusions. A reproducible generalized or focal arthritis, synovitis, periostitis and tendonitis has been induced in rats by intracutaneous injections of a Freund-type adjuvant which was most potent when macerated striated muscle tissue was added to the inoculum. The pathogenetic mechanisms underlying the production of these changes is at present unclear.

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Plasma Thromboplastin Component: Influence of Coumarin Compounds and Vitamin K on Its Activity in Serum. (22180)

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A new blood clotting factor, termed plasma thromboplastin component (PTC), was recently described by Aggeler, *et al.*(1) and subsequently called Christmas factor by Biggs, *et al.*(2). Little is known about the formation of PTC or about its disappearance from the blood stream. Early workers demonstrated that the level of prothrombin in the blood stream is influenced by vit. K and the coumarin compounds. Since these compounds have been shown to influence "stable factor" as well(3,4), it is of interest to determine whether PTC is similarly affected. The present study demonstrates that patients deficient in vit. K develop a depression of PTC, and confirms the observation of Sise, *et al.*(5) that following the administration of the coumarin compounds there is also a depression of PTC. In both groups we find that PTC activity is restored, in part at least, by the administration of vit. K analogues.

Methods. *One-Stage Prothrombin Time* was determined by the Link-Shapiro(6,7) modification of Quick's method(8), using commercial rabbit brain thromboplastin* (normal: 14 seconds). *One-Stage Prothrombin Activity* ("per cent" of normal). Normal oxalated plasma was serially diluted with saline (90%, 80%, etc.), and the one-stage prothrombin times of various dilutions determined. Observed times were then plotted against dilution, to provide a standardization curve for comparison of unknown plasmas with normal ones. *Assay of Hemophiloid Factors; Principles Involved:* Thromboplas-

tin is essential for normal conversion of prothrombin into thrombin. Thromboplastin obtained from rabbit brain will react with normal recalcified plasma to form a clot in 14 seconds. This is the basis of the one-stage prothrombin time test. Washed human platelets can form an equally potent thromboplastin, but unlike rabbit brain, must first be incubated with the hemophiloid factors and calcium(9). Hemophiloid factors include: plasma thromboplastin component (PTC), plasma thromboplastin antecedent (PTA) (10) and anti-hemophilic factor (AHF). The ability of these factors, in combination, to transform platelets into a potent thromboplastin, the equivalent of that obtained from rabbit brain, is the basis for the thromboplastin generation test, Biggs, *et al.*(9,11). In the present experiments the thromboplastin generation test is modified so that it becomes a quantitative rather than merely a qualitative assay. Thus the relative levels of any one of the hemophiloid factors can be determined. In the quantitative procedure a reaction mixture containing platelets and calcium is supplied with two of the hemophiloid factors, each in high titer. The third factor—the one to be assayed—is added as an unknown or test preparation. A deficiency of this third factor will be demonstrated by faulty "activation" of thromboplastin. Of the three possible assay procedures, only one was used in the present study—the assay for PTC. *Details of the PTC Assay:* A standard platelet suspension is obtained by the differential centrifugation of citrated normal

*Prepared by Warner-Chilcott Laboratories.

human plasma(9). Adsorbed plasma is prepared by adding 0.2 cc aluminum hydroxide suspension(12) to 2.0 cc of citrated human plasma, agitating for 5 minutes at 37°C, and centrifuging at 2500 rpm for 15 minutes. The supernatant plasma is then removed and diluted 1:5 with 0.85% saline. Serum is prepared by incubating clotted human blood at 37°C for 90 minutes. To insure uniformity, reagents are then frozen and stored at -40°C until used. Sera provide the PTC and PTA needed to activate the platelet suspension; adsorbed plasma provides the AHF. A test serum under investigation, or a normal control serum, is the source of PTC. The assay is based upon variations in PTC activity supplied by such sera. These sera also contain PTA, but unknown sera cannot be used as a reliable source of this factor, since PTA levels in them may vary. We therefore make it a practice to add a stabilizing surplus of PTA, in the form of serum from a patient rich in PTA but congenitally deficient in PTC. Finally, a stabilizing amount of AHF is likewise supplied by adding normal plasma, adsorbed with $\text{Al}(\text{OH})_3$; by virtue of the adsorption, this plasma is almost entirely free of PTC and prothrombin.

The reagents are then mixed. An appropriate amount of saline is included in Sol. A to increase sensitivity of the assay.

Sol. A: .10 cc test serum (unknown or normal control serum); 8.20 cc .85% saline; .46 cc serum from patient congenitally deficient in PTC but rich in PTA; 1.24 cc 15% calcium-free acacia in .85% saline to maintain colloidalty.[†]

Sol. B: .20 cc normal $\text{Al}(\text{OH})_3$ adsorbed plasma, diluted 1:5 with .85% saline; .20 cc human platelet suspension; .20 cc .02 M CaCl_2 . Incubate 0.20 cc of Solution A with .60 cc of Solution B in a test tube at 37°C. In this mixture, the activity of thromboplastin reaches a peak after 3-6 minutes of incubation; it then diminishes. Since the amount of PTC in the incubation mixture limits the level reached by this peak, the peak level was chosen as an appropriate measure

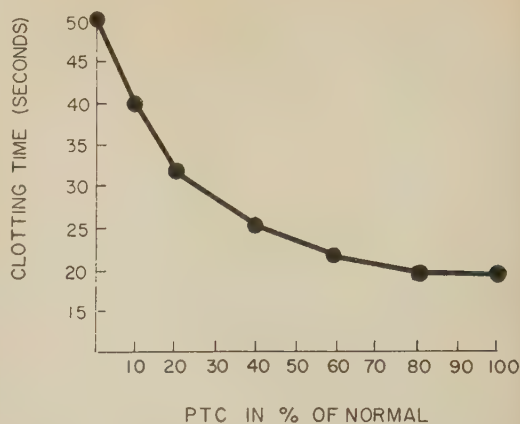


FIG. 1. Standardization curve of PTC assay. The assay is standardized by supplying PTC in graded amounts in the form of serially diluted normal serum.

of PTC. To detect the topmost level with certainty, determinations of thromboplastic activity are made periodically in the region of the peak. At one minute intervals, 0.10 cc aliquots of the incubating thromboplastin are added, simultaneously with 0.10 cc of 0.02 M CaCl_2 , to 0.10 cc of normal citrated plasma and the clotting times recorded. The citrated normal plasma provides labile and stable factors, prothrombin and fibrinogen. The shortest clotting time of the mixture—recorded at the moment of maximal activation—provides a measure of peak thromboplastic activity. It is recorded as the measure of PTC.

A PTC standardization curve was prepared for the comparison of PTC content of unknown and normal sera. This was accomplished by supplying PTC to the assay in graded amounts in the form of normal serum, serially diluted. The clotting time at each dilution was plotted in per cent of normal (Fig. 1). Such a curve must be prepared for each new set of reagents. By means of the curve, the clotting times of unknown sera can be interpreted in per cent of normal PTC activity.

Results. The activity of PTC in coumarin-treated and vit. K-deficient patients was studied by means of the PTC assay. The well known deficiency of prothrombin and stable factor which these patients develop was measured by the one-stage prothrombin

[†] Acacia was later found to be unnecessary and was replaced by saline.

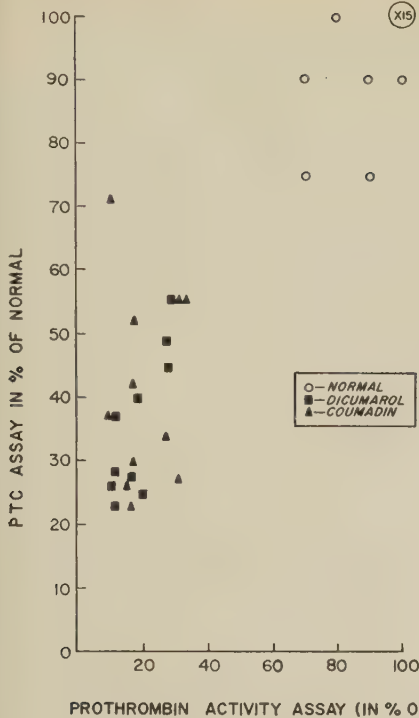


FIG. 2. Relationship between coumarin anticoagulation and PTC activity in 42 treated and untreated patients.

time test and the prothrombin time activity test. These latter assays are not influenced by variations in PTC, since the thromboplastin used in them is fully "activated" already.

Effect of 4-Hydroxy Coumarin Compounds on PTC Levels: Blood specimens were collected from 20 normal persons. A one-stage prothrombin activity test and PTC assay were done on each sample. These same studies were undertaken on 21 patients to whom either 3-3'-methylenebis (4-hydroxy coumarin), known as Dicumarol, or 3 (α -phenyl- β -acetyethyl)-4-hydroxy coumarin, known as Coumadin, had been administered for 4 or more days. These latter patients were suffering from either thrombophlebitis or myocardial infarctions. In Fig. 2 are recorded the test results obtained in both normal and treated patients. PTC levels are plotted against one-stage prothrombin activities. The Dicumarol-treated patients demonstrated significant deficiencies of PTC as well as a de-

pression of the prothrombin activity test. Only one Coumadin-treated patient failed to demonstrate a severe deficiency of PTC. In a second group of patients there was an opportunity to study clotting factor levels before as well as during Dicumarol administration. Representative data, collected from a patient with thrombophlebitis, are shown in Fig. 3. A progressive depression of one-stage prothrombin activity, reflecting drug effect, was accompanied by a similar depression of PTC activity.

Effect of Vit. K_1 on a Depression of PTC Induced by Dicumarol: In patients treated with Dicumarol an attempt is made to maintain the one-stage prothrombin activity between 18% and 30% of normal (27-20 sec.) In one such patient, the drug dosage was inadvertently increased over a 3-day period so that the prothrombin activity decreased to 9% of normal and PTC activity to 34% of normal. At this time, 50 mg of vit. K_1 was given intravenously as a corrective measure. Six hours later, the prothrombin activity had risen to 28% of normal and the PTC activity

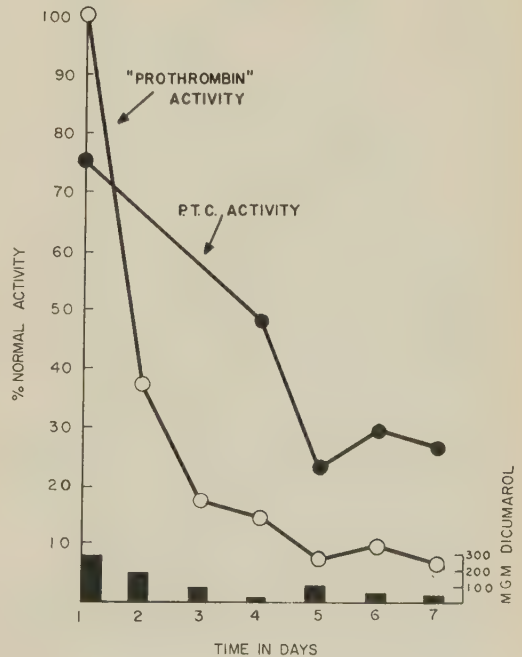


FIG. 3. Effect of Dicumarol on PTC levels and on the one-stage prothrombin activity test in a single patient.

to 43% of normal. After 18 hours, the results were 45% and 57% respectively.

Fat Maladsorption Syndrome: A child, aged 2 years, was found to be unable to adsorb fat-soluble vitamin K. This patient had cystic fibrosis of the pancreas. When tested she demonstrated a marked prolongation of the one-stage prothrombin time (58 sec., or 7% of normal) and a marked deficiency of PTC (8% of normal). Then 15 mg of menadione sodium bisulfite were administered intramuscularly. One hour later, the clotting factor activities had not changed. Eighteen hours after the vitamin administration, the prothrombin time was 18 seconds (57% of normal) and the PTC activity was 65% of normal.

Discussion. Deficiencies of prothrombin, fibrinogen, labile factor and stable factor can be congenital in origin; they can also reflect a vitamin deficiency or be manifestations of hepatic injury. In this regard, plasma thromboplastin component (PTC) is not different. Both vit. K and its coumarin antagonists exert a measurable influence on the activity of PTC, comparable to their effect on prothrombin and stable factor. This influence has been previously investigated, but with the exception of Sise *et al.* (5) the technics employed were too insensitive to demonstrate the moderate alterations that occur (1,13,14).

It is clear that stable factor and PTC have many common properties and characteristics. The one-stage prothrombin time test seems to present the strongest evidence for regarding these two factors as distinct. This test detects deficiencies in prothrombin, stable factor, labile factor and fibrinogen but not in PTC. The rabbit brain used as thromboplastin supplies a PTC-like activity artificially. However, human platelets when used as thromboplastin do not supply PTC-like activity. The necessary "activation" of human platelets by PTC and the other hemophiloid factors makes assay of these factors possible. With further investigation, PTC

may prove to be as biologically important as are prothrombin and stable factor.

Summary. Deficiencies of plasma thromboplastin component (PTC) are not necessarily congenital in origin. Serum levels of PTC were depressed following the administration of two 4-hydroxy coumarin compounds to 24 patients. PTC activity was also reduced in a patient who developed a deficiency of vitamin K subsequent to an inability to adsorb fats (cystic fibrosis of the pancreas). In both groups of patients, PTC activity rapidly increased towards normal following the administration of vit. K analogues.

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Lipemia and Glomerular Lesions in Rats Fed Diets Containing N-N'-Diacetyl- and 4, 4-4', 4'-Tetramethylbenzidine. (22181)

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While testing a variety of chemical compounds for their ability to induce tumors in Buffalo-strain rats(1), it was observed that both N-N'-diacetylbenzidine and 4,4-4',4'-tetramethylbenzidine caused severe lipemia accompanied by a characteristic renal lesion. The rats given these drugs died from the effects of the drugs before the time when induced tumors ordinarily appear following exposure to a carcinogen.

Methods: The benzidine compounds were dissolved in the propylene glycol portion of the diet incorporated at equal molar levels of 0.25 and 0.27% respectively into diet #222,* an otherwise excellent diet composed mostly of natural foodstuffs. This was fed to 5 groups of rats and 2 groups of inbred mice.

Results. No difference in the effects of the 2 compounds was observed. After 2 to 4 months on the regimen, the rats lost weight rapidly. During the terminal week of life, the loss often amounted to 20-50 g for individual rats. Fifty rats were autopsied. When killed during the terminal period, the blood was pale pink and clotted quickly. Generalized edema with ascites and hydrothorax was usually present and the kidneys were pale brown with slightly granular surfaces.

Frozen sections were made from the brain, liver, and kidney of 10 rats and stained by Sudan III. Large and small globules of bright red material, indicating the presence of fat were found in the vessels. This material was especially abundant in the glomerular capillaries (Fig. 1).

Tissues taken at autopsy were routinely fixed in Zenker-formol, embedded in paraffin,

and stained with hematoxylin and eosin. Selected kidney sections were also treated by the periodic acid-Schiff and the Wilder reticulum method, Masson's trichrome stain, and toluidine blue.

Only an occasional glomerulus was altered in rats killed early, but changes were regularly found in the glomeruli of rats surviving 3-4 months (Fig. 2). The lesion was characterized by vacuoles within the glomerular loops. Bowman's space was often obscured, and there appeared to be adhesions between the layers of Bowman's capsule. The tubular cells were little altered, though many hyaline and waxy casts were present in the

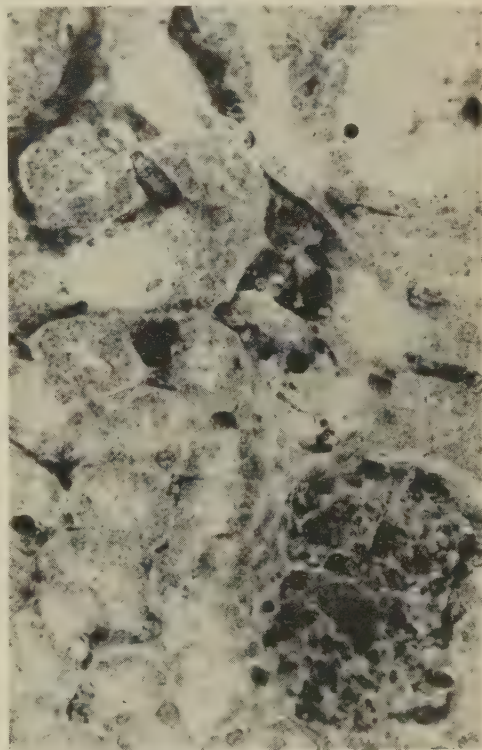


FIG. 1. Glomerular capillaries filled by lipid, which also appears within intertubular vessels. Sudan III. 280 $\times$.

* Composition of diet No. 222: Commercial casein 3, skim milk powder 22.75, whole wheat meal (hard spring) 60.52, debittered brewer's yeast 1, whole liver powder (Wilson's) 1, NaCl 1.4, iron citrate .13, cod-liver oil 2, corn oil 5.2, propylene glycol 3.

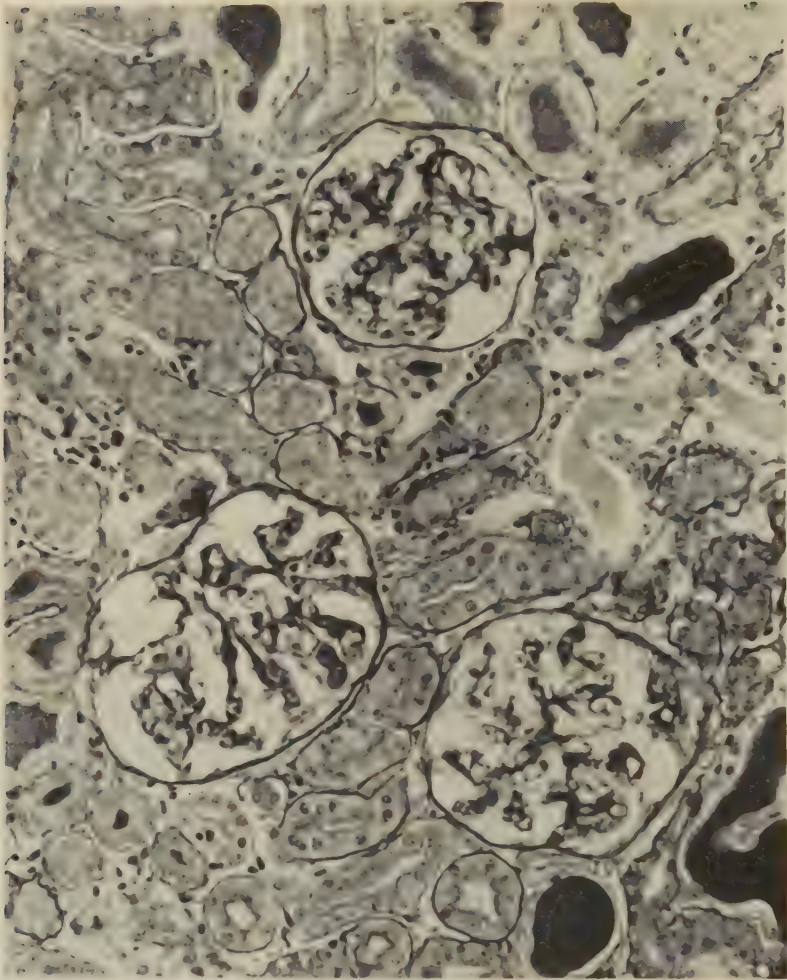


FIG. 2. Three glomeruli, showing small cystic dilatations, and obliteration of Bowman's space. Tubular cells appear normal, but heavily stained casts are present in the lumens. Periodic-acid Schiff. $\times 280$.

tubular lumens. A large number of control animals examined showed none of the changes described.

The mice showed no effects from ingesting the drug; and only those lesions usually found in untreated mice of the strains tested, C3Hb/HeN and A, were observed at autopsy.

Discussion. The toxic effect of the 2 benzidine compounds at the doses given was so severe for rats that the animals failed to survive the period usually required for the induction of tumors. Consequently no appraisal can be made as to the carcinogenicity of these compounds.

Kidney lesions similar to this, to our

knowledge, have never been observed in untreated rats. However, kidney lesions having many of the same morphologic features have been described by Wilens and Stumpf (2) in rabbits treated with cortisone. The initial and constant alteration of the rabbit kidney lesion was a moderate and fairly uniform dilation of almost all glomerular capillaries. The rabbits also had an extreme degree of lipemia, and Wilens and Stumpf suggested that the altered proportions of the blood lipids might cause protracted periods of glomerular capillary stasis. This effect of cortisone has not been found in other species, including man. The dilated capillaries noted

in the rats receiving the drugs appeared to be empty after paraffin embedding, but were shown to be filled with fat in frozen section preparations. The small number of erythrocytes in the blood plasma in the rats and the filling of the lumens with fat may be a reason why the dilated capillaries appeared empty after paraffin embedding.

Summary. Lipemia and a glomerular lesion consisting of many fat filled spaces in the glomerular tuft appeared in from 2-4.5

months in Buffalo-strain rats fed diets containing N-N'-diacetylbenzidine and 4,4-4', 4'-tetramethylbenzidine. No similar lesions were found in two strains of mice ingesting diets containing these two chemicals.

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Effect of Changes in Environmental Temperature on Stomach Emptying in Rats.* (22182)

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Rats transferred from room temperature to cold environment lose weight during the first few days. One obvious reason seems to be that, in spite of the increased caloric requirements in cold, the animals do not consume more food during this time than they did at room temperature. In search for the cause of this unsatisfactory food intake in experiments reported below we investigated specifically the question whether gastric emptying time is influenced by changes of environmental temperature. Although it has been shown earlier (1) that stimuli for food intake do not originate from the gastro-intestinal tract, everyday experience indicates that disturbances in physiological functions of the digestive organs may interfere with voluntary food intake. An early and important phase of food utilization is emptying of the stomach. We, therefore, considered first the changes in stomach emptying of animals exposed to lower temperature. The effect of changes in environmental temperature on stomach emptying has been discussed earlier(2) but the conclusions were equivocal. We decided, therefore, to investigate this problem directly applying a method for determination of stomach emptying time

which proved to be valuable in our earlier investigations(3). The method is based on determination of N found remaining in the stomach 2 hours after voluntary consumption of a measured quantity of protein.

Methods. Male, Sprague-Dawley rats of 110-220 g weight were used. Stomach emptying time was determined by a method based on the method of Cori(4). However, we found, in preliminary experiments, that stomach emptying time varied widely after forced feeding by stomach tube. Excitement of the animals may be responsible for these variations. Consequently, adult male rats were trained to eat, spontaneously, definite quantities of protein after 24 hours starvation. Animals were maintained at room temperature of $22.2^{\circ}\pm 0.5^{\circ}\text{C}$. After 24 hour fast, with free access to water, each animal was presented with 2,-2,2 g of low fat tuna meat.[†] The N content of each batch of tuna meat was determined and the amount offered was calculated so that approximately 86 or 100 mg was consumed by the animal. Only those animals which consumed all of the tuna without spillage, within 15 minutes, were used for the experiments. After a digestion period of 2

* These investigations have been supported by Contract USAF Arctic Aeromedical Laboratory.

[†] "Chicken of the Sea" brand Dietetic Tuna was used after draining of the cans.

hours, with free access to water, they were sacrificed by a blow on the head. The abdomen was opened immediately and ligatures were placed at the cardio-esophageal, gastroduodenal and ileo-cecal junctions. Stomach and small intestines with their contents intact were then removed, and after opening the lumen the contents were rinsed with distilled water into separate beakers. These washings were transferred to 100 ml volumetric flasks containing 10 ml concentrated sulfuric acid. The flasks were then placed in a boiling water bath for 90 minutes to completely disintegrate all proteinaceous material. After this digestion, samples were diluted with water to 100 ml volume. The N was determined on an aliquot part after further digestion with sulfuric acid, either by nesslerization using a Lumetron photocolormeter, or by semi-micro Kjeldahl method. Both methods gave closely comparable results. The same procedure was followed with animals subjected to cold and heat with the following alterations: *For acute cold studies* immediately after all the tuna was eaten at room temperature, the cage with the animals was placed in a large walk-in refrigerator at 3°C for the 2-hour digestion period. *For acute heat studies*, immediately after all tuna was eaten at room temperature, the cage with the animals was placed in a dry heat room, temperature 37.8°C for the 2-hour absorption period. *For studies with cold adaptation* 5 days prior to start of experiment, animals were placed in the refrigerator at 3°C. They received food (Purina Chow) and water *ad lib.* for 5 days. The sixth day each animal was fasted 16 hours. This shorter fasting time was chosen to compensate for the increased caloric demand in the cold, and because we had found previously, that stomach and intestines were entirely emptied after 16 hours of fasting in the cold. After the 16-hour fast each animal was presented with tuna and remained at 3°C for 2 hours after consumption of food; they were then removed and sacrificed instantly. *Heat adaptation studies*, 5 days prior to start of experiment, animals were placed in a dry heat room with temperature 37.8°C. They received food (Purina Chow) and water *ad lib.* for 5 days.

On sixth day animals, after 24-hour fast, were presented with tuna meat. They remained at 37.8°C for 2 hours after consumption of food and thereafter were removed and sacrificed instantly. In 3 animals stomach emptying was investigated after only one-day heat adaptation.

Results of our experiments are condensed in Table I. These data show that at normal room temperature, about 87% of the consumed N leaves the stomach within 2 hours. These values agree very well with our earlier findings. In animals exposed to *cold* after consumption of food, the amount of food left in the stomach after 2 hours was much larger. In the first 4 rats only about 63% and in the 8 rats belonging to another batch of animals, only 27% of the protein had left the stomach. We cannot account for the difference in the reaction of the 2 groups of cold exposed animals, but inspection of the data and statistical evaluation show that emptying of stomach was significantly delayed on acute exposure to cold in both groups. Consequently, we repeated the experiment with animals which were adapted to cold. The results in these rats showed that stomach emptying in animals adapted to cold was identical with that observed in normal rats at room temperature.

In the next group of experiments, animals were transferred to the hot room after consumption of food. Stomach emptying was even more delayed than after exposure to cold. In some cases the N content of stomach after 2 hours was actually higher than the amount consumed. This might indicate that a profuse gastric secretion was induced by heat, and the N content of this secretion was responsible for the increased amount of N found in the stomach(5). Further experiments on animals which had been acclimatized to heat for 5 days show that similar to adaptation to cold, adaptation to heat restores the stomach emptying to normal values. We do not know how fast adaptation occurs but the few experiments performed to date (Nos. 52-54) show that a 24-hour exposure to heat is not sufficient to restore stomach emptying time to a normal value.

To determine whether gastric digestion has

TABLE I. Influence of Environmental Temperature on Elimination of Dietary Protein from Rat Stomach.

Exp. condition	— 2 hr after food consumption —			
	mg N consumed	mg N in stomach	mg N in intestines	Avg % of N emptied from stomach
At room temp.	86	6.7	14.5	91.8 $\pm$ 2.75
	"	6.4	14.7	
	"	5.3	14.2	
	"	10.3	14.4	
	100	8.7		85.8 $\pm$ 3.86
	"	16.3		
Acute cold exposure	"	18.6		
	"	14.		
	86	25.7	11.6	63. $\pm$ 6.48
	"	29.6	12.3	
	"	38.9	12.4	
	"	33.9	7.5	
	"	69.0		
	"	61.5		
	"	60.5		
	"	60.0		
	"	62.5		27. $\pm$ 4.12
	100	74.		
	"	83.		
	"	65.		
After 5 days cold adaptation	"	69.		84.9 $\pm$ 8.10
	86	15.9	14.6	
	"	14.0	18.2	
	"	5.0	15.3	
	"	10.1	12.2	
	100	21.3		
	"	7.3		
	"	24.5		
At room temp. after 5 day cold adaptation	"	15.0		30. $\pm$ 7.84
	"	26.7		
	86	12.6	12.4	
	"	14.6	18.6	
Acute heat exposure	86	55.0	13.0	0.
	"	57.0	16.8	
	"	68.0	17.0	
	"	54.0	12.2	
	"	67.0	11.4	
	100	100.0		
	"	116.0		
	"	131.5		
5 day heat adaptation	"	132.5		83. $\pm$ 5.68
	"	133.5		
	100	14.3	17.8	
	"	18.8	17.6	
	"	21.9	16.4	
	"	26.2	15.8	
	"	15.4		
	"	22.2		
After 24 hr heat adaptation	"	12.0		12.2
	"	8.5		
	"	15.4		
	86	48.0	8.4	
	"	54.5	14.6	
	"	64.5		

been disturbed by acute changes of environmental temperature, we investigated the N present in intestinal contents 2 hours after food consumption. These experiments were based on earlier investigations in which we found that hard to digest proteins such as zein, raw eggwhite or certain soy protein preparations are emptied from the stomach at the same rate as easily digestible proteins. The intestines contained, however, much larger quantities of proteinaceous material 2 hours after feeding of zein, raw eggwhite or soy protein, than after consumption of casein or fish protein(3).

Table I shows that intestinal N content in cold and heat exposed animals was very similar to the value observed in rats at room temperature. These results seem to suggest that changes in gastric motility and not disturbed digestion may be primarily responsible for the delay in stomach emptying after acute temperature changes.

Discussion. The experiments reported above show that following a protein meal, sudden transfer of rats from room to either high or low environmental temperatures, prolongs the gastric emptying time. The question whether decreased motility, closure of the pylorus or disturbance in gastric digestion or a combination of these factors is responsible for this effect can not be definitely answered by our experiments. It seems, however, that changes of motility are the main cause. This conclusion is based on extensions of earlier experiments in which gastrointestinal propulsion of hard to digest proteins, such as zein, or raw eggwhite was studied. In those experiments stomach emptying was normal, but the intestines contained abnormally large quantities of undigested nitrogenous material (4). The finding that distribution of N be-

tween stomach and intestines of cold or heat exposed animals, is the same as that observed in control experiments, seems to indicate that disturbances of gastric digestion may not be the primary cause of the observed heat and cold effects. Our experiments indicate that after adaptation to changed environmental temperature, stomach emptying time is again similar to that observed at room temperature. We must, therefore, conclude that neither cold nor hot environmental temperature as such, but rather the sudden change of environmental temperature is responsible for the delayed stomach emptying. The common factor involved seems to be an increased epinephrine production which delays gastric emptying time. Epinephrine is produced, we found earlier, in abnormally large quantities during sudden cooling as well as sudden exposure to heat, but is not present in increased amounts after adaptation(6,7).

Summary. 1. Transfer of rats from room (22°C) to either low (3°C) or high (37°C) environmental temperature immediately following a protein meal delays emptying of stomach. 2. After adaptation to changed surrounding temperature stomach emptying was found to be again similar to that observed at room temperature.

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Effects of Intravenous Infusion of Experimental "Instant" Fat Emulsion Into Volunteer Subjects.* (22183)

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At the first *ad hoc* conference held by the National Research Council dealing with intravenous fat emulsions the stability and toxicity of such newly developed preparations were discussed(1). Except for variations in the type of fat (coconut oil, corn oil, sesame oil, etc.) and the character of the emulsifier ionic or nonionic, all investigators employed practically the same methods of preparation (2,3). The accepted procedure involved a high speed premix of constituents oil, emulsifier and water to form a crude blend after which followed mechanical pressure homogenization and finally sterilization by autoclaving. While these freshly prepared emulsions were well tolerated intravenously, administration of emulsions aged more than a month was correlated with a significant increase in reactions believed to be due to lipolysis, hydrolytic rancidity or physical alterations in particle size(1).

The present investigation was undertaken with a new type of intravenous fat emulsion prepared by means of special emulsifiers which make possible "instant" or spontaneous emulsification of oil and water mixtures for immediate intravenous administration. By this method the effects of aging on the emulsion with resultant toxic degradation products seemingly would be obviated. This study is concerned with the use of one such "instant" fat emulsion tested intravenously in experimental animals and in volunteer human subjects.

Method of study. The emulsion was prepared as follows. Fifteen g of sterile distilled whole coconut oil and 1 g of special emulsifier were placed in small intravenous bottles (250 ml). The emulsifier known as Drew #535

consisted of glycerol and polyglycerols partially esterified with mixed hydrogenated and unhydrogenated soy bean fatty acids. After addition of oil and emulsifier the bottles were capped with transfusion type rubber stoppers and autoclaved at 20 lb pressure for 35 minutes. After sterilization, these bottles were stored at room temperature. Before each infusion a bottle of the oil mixture was warmed and 10 ml of absolute alcohol was added under sterile conditions through the inflow hole. An almost clear solution formed upon mixing. Finally 125 ml of sterile 5% glucose in water was added to yield, after moderate shaking, an opaque emulsion. The latter was administered promptly by venoclysis through transfusion equipment containing a strainer-filter. The "instant" emulsion appeared uniformly white, flowed freely and remained stable for almost 5 hours. Occasional shaking prevented any tendency for separation. Caloric value approximated 230 calories for 150 ml. Microscopic examination with ocular micrometer under dark field illumination revealed active Brownian movement with 70% of the particles about 1 μ and the remainder up to 5 μ in size. The pH of emulsion was 7.35, refractive index 1.345, and dispersion a narrow spectrum band. The "instant" fat emulsion was first administered into a group of 10 dogs. Each animal received daily an infusion of 75 ml of the emulsion administered usually at the rate of 40 drops (2 ml) per minute. Observations were limited to temperature changes recorded at hourly intervals and to such visible reactions as vomiting, chills, etc. Following the animal studies, the same kind of emulsion was tested in a group of volunteer hospital patients. With each subject in the postabsorptive state, 150 ml of the emulsion was infused 20 to 30 drops (1 ml to 1.5 ml) per minute. The temperature, pulse, respirations and blood pressure were taken at hourly

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TABLE I. Reactions of Dogs and Human Subjects to Intravenous Infusion of "Instant" Fat Emulsion. 15 infusions in 10 dogs; 148 infusions in 46 humans.

Reactions	Animal	Humans
	%	
Minimum temp. 0-1°F	43	63
Moderate " 1-2°F	33	32
Maximum " 2°F	20	5
Vomiting	26	8
Chills	13	6
Diarrhea	20	2
Excessive salivation	13	—
Headache	—	9
Dyspnea	7	2
Sweating	—	6
Back pain	—	10
Chest pain	—	1

intervals for the first 12 postinfusion hours of a 24-hour study. Complete blood counts, sedimentation rates, hematocrits and standard urinalyses were performed by the usual technics at regular intervals. In several subjects lipid chemistry of blood was determined quantitatively for rate of clearance of fat from the circulation(4).

Results. Five dogs received single infusions of 50-75 ml; while 5 other dogs received 100 ml emulsion divided into 2 daily doses. The results have been recorded in Table I.

A total of 148 intravenous infusions of the fat emulsions were administered into human subjects. In this group of 46 volunteers, 28 received single infusions while 3 or more infusions were given to other 18 volunteers. The temperature changes associated with these injections and other reactions have also been incorporated in Table I.

Temperature elevations following animal and human infusions were always of a tem-

porary nature and subsided rapidly to preinfusion level. Where multi infusions were given, temperature response varied from maximum elevation to moderate or minimal reaction with each infusion test. Constitutional reactions, when considered singly, totalled 44% but actually averaged 28% because in many cases these untoward effects were multiple in character; *viz.* vomiting, chills, etc. Changes in blood pressure during infusion and in the postinfusion phase were variable and showed no significant trend. Red blood cell counts, hematocrit cell volumes and sedimentation rates did not deviate markedly from normal. In 40% of white blood counts, the first postinfusion blood sample indicated a leukocytosis which averaged 19000 white blood cells after which there was a rapid return to preinfusion level which averaged 10200 white blood cells. Chylomicron counts increased markedly during infusion and then declined rapidly, usually within 4-5 hours after completion of infusion in a manner similar to the chylomicron pattern observed with other fat emulsions. Total blood lipids and partition fractions showed no characteristic trends (Table II). Postinfusion urine samples failed to reveal abnormal excretion of fat or other pathologic changes referable to the use of the emulsion.

Discussion. Considerable data have been reported on effects of "standard" type of fat emulsion but there has been no report with the present preparation. Our data have shown that infusions of the "standard" 10% fat emulsion in units of 1 liter were well tolerated in 77% of recipients when adminis-

TABLE II. Laboratory Data Pertaining to the Single Infusion of 125 ml of 15% "Instant" Fat Emulsion into Subject, A.S.

Infusion	Temp., °F	Blood pressure, mm Hg	Pulse rate, min.	R.B.C. count (million)	W.B.C. count (thous.)	Hematocrit, %	Sedimentation time, mm/hr	Chylomicron count	Total lipids, g/%	Phospholipids, mg/%	Total cholesterol, mg/%	Cholesterol esters, mg/%	Neutral fat, mg/%
Pre	98.8	140/88	86	4.26	9.8	46	28	4	.63	160	123	83	84
End of	99.0	160/88	98	4.24	11.0	40	36	108	.88	180	150	114	273
2 hr	102.8	160/90	110	4.41	11.2	40	36	86	.83	190	152	106	211
5 "	101.2	148/90	100	4.38	10.6	44	30	52	.75	190	157	119	123
7 "	100.6	145/90	94	4.29	10.6	44	34	58	.71	173	144	105	120
18 "	99.0	150/88	94	4.32	10.8	46	34	20	.79	165	152	106	196
24 "	99.0	154/80	90	4.38	10.2	48	20	6	.48	170	140	100	80

tered as a freshly made preparation but toleration declined to 69% when emulsions were 5 to 10 weeks old. A significant rise in concentration of free fatty acids and unstable peroxides or increase in size of fat particles up to 25 μ has been described as characteristic of aging emulsion. Accumulation of any of the latter products might be related to toxicity and thermogenicity of the emulsion when used for intravenous alimentation. These reactions may be compared to toxic effects produced experimentally by injection of free short chain fatty acids(5) or to pulmonary and pyrogenic effects associated with trapping of particulate matter in the lungs(6). Stability of an artificial emulsion should be considered to be a function of time during which physical alterations in size of fat particles and chemical interactions between water and fat may occur. A technic for rapid emulsification of oil and water mixtures with elimination of the factor of aging would therefore be warranted. Although the present "instant" fat emulsion cannot now be considered to be a suitable preparation for general clinical use,

investigations along the lines described in this report have been continued.

Summary. An "instant" fat emulsion prepared by manual mixing of oil, emulsifier, alcohol and water was developed and tested. This type of emulsion obviated high pressure homogenization and was freshly prepared for each infusion. A total of 63% of the infusions were fairly well tolerated while in the other 37% reactions were observed.

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Modification of Tumor-Response to Rous Sarcoma Virus (RSV) by Hydrocortisone.* (22184)

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It is well known(1) that cortisone and hydrocortisone usually depress resistance of laboratory animals to viral infection and that effective doses of these steroids are frequently associated with evidence of increased multiplication of the virus. On the other hand, cortisone and several related steroids beneficially affect certain transplanted lymphosarcomas and leukemias in mice(2). However, rabbits

injected with cortisone developed much larger tumors upon inoculation with rabbit fibroma virus although tumor size was not appreciably affected if injections of cortisone were delayed until appearance of the tumor(3). Data presented here show that (a) when Rous sarcoma virus (RSV) was inoculated into chicks pretreated with large doses of hydrocortisone, tumors appeared later and were greatly altered in appearance; (b) when hydrocortisone was discontinued the altered tumors promptly reverted to typical invasive type; and (c) when lower doses of hydrocortisone were employed or when injections of hydrocortisone were delayed until 2 days after in-

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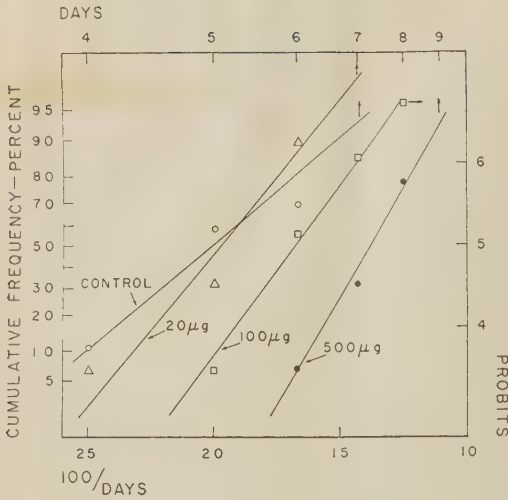


FIG. 1. Effect of pretreatment with hydrocortisone on latent period of RSV.

oculation of RSV the latent period was not affected; tumors which appeared were typically invasive but increased in size at a rate which was appreciably more rapid than that of control chicks.

Materials and methods. RSV (also referred to as chicken tumor I agent) was maintained by serial passage in chickens. A single lot (CT 581) of frozen standard RSV, prepared by differential centrifugation and preserved at -70°C as previously described (4), was used throughout. The diluent for standard RSV was saline containing 2% inactivated (56°C for 30 minutes) normal horse serum. Unsexed white Leghorn chicks under 7 days of age were used. Two tenths ml of suitably diluted RSV were inoculated into the subcutaneous tissue of left wing web. Ventral surface of the wing was swabbed with isopropanol and the hypodermic needle was inserted through the muscle into subcutaneous tissue of wing web, thus avoiding leakage. Each bird was examined daily by swabbing the wing with isopropanol and examining the wing with both direct and transmitted illumination. Hydrocortisone was suspended in the usual diluent (5) and 0.5 ml of the suspension were injected intramuscularly into the thigh muscle. Control birds received daily injections of diluent alone. Data were analyzed as previously described (6,7) using probit analysis (8).

Results. Fig. 1 shows effect of various doses of hydrocortisone on latent period of chicks inoculated into the wing web with RSV diluted 10^{-2} . Daily intramuscular injections of hydrocortisone were begun 2 days before inoculation of RSV and continued throughout the experiment. Control and treated groups consisted of 33 chicks each. It is clear that the latent period was prolonged in birds injected daily with 500 or 100 μg of hydrocortisone. However, the latent period in birds injected with 20 μg of hydrocortisone was not affected, but tumors which appeared were much larger than those in control birds.

In the above experiment the most prominent feature observed was the fact that all tumors which appeared in birds injected with 500 μg of hydrocortisone were grossly, markedly altered in appearance. In contrast to the usual rapidly growing, soft invasive tumor, the tumors which arose in hydrocortisone-treated birds were firm, sharply circumscribed and grew much more slowly. Characteristics of these altered tumors are summarized in Table I and photographs of typical and altered tumors are shown in Fig. 2-4.

Table II summarizes data showing the effect of daily dosage and dosage schedule on latent period and incidence of altered tumors. Altered tumors were produced only in chicks previously injected with large doses (500 $\mu\text{g}/\text{chick}$, i.e. 10 mg/kg) of hydrocortisone. Indeed, when dosage was decreased or delayed until after inoculation of RSV the tumors were not only typical and invasive but also were obviously larger and grew more rapidly

TABLE I. Characteristics of Tumors Produced by RSV* in Hydrocortisone-Treated and Control Chicks.

Characteristic	Control	Hydrocortisone-treated
Latent period†	4.6 days	7.6 days
Site of developing tumor	Wing web	Muscle
Type	Multiple	Single
Rate of growth	Rapid	Slow
Type of "	Diffuse & invasive	Spherical, sharply circumscribed
Consistency	Soft	Firm

* Standard RSV diluted 10^{-3} .

† LP₅₀ or time in days to produce tumors in 50% of the chicks.

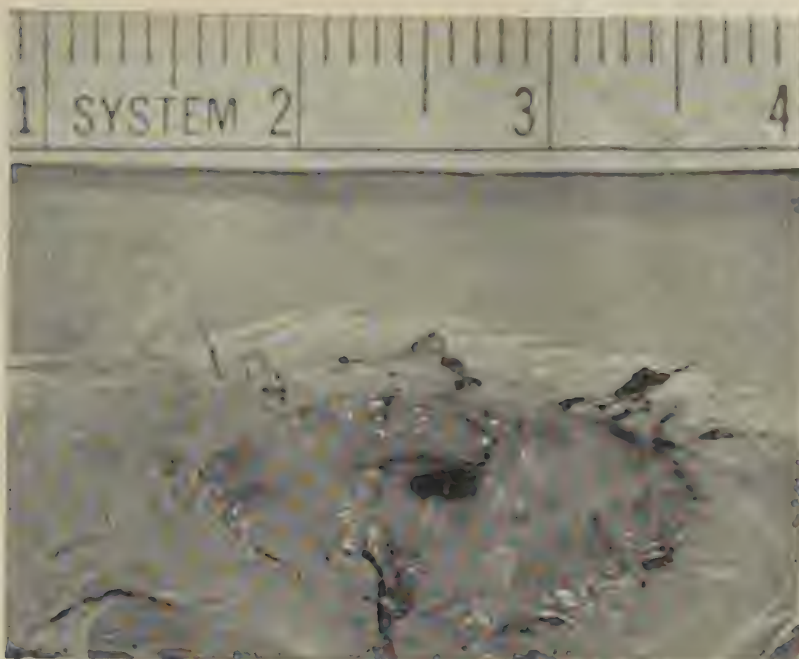


FIG. 2. Control tumor in wing. Twenty-one days after inoculation of RSV diluted 10^{-1} . Scale metric system.



FIG. 3. Altered tumor produced by pretreatment with hydrocortisone. Front (ventral) view. Twenty-one days after inoculation of RSV diluted 10^{-1} .

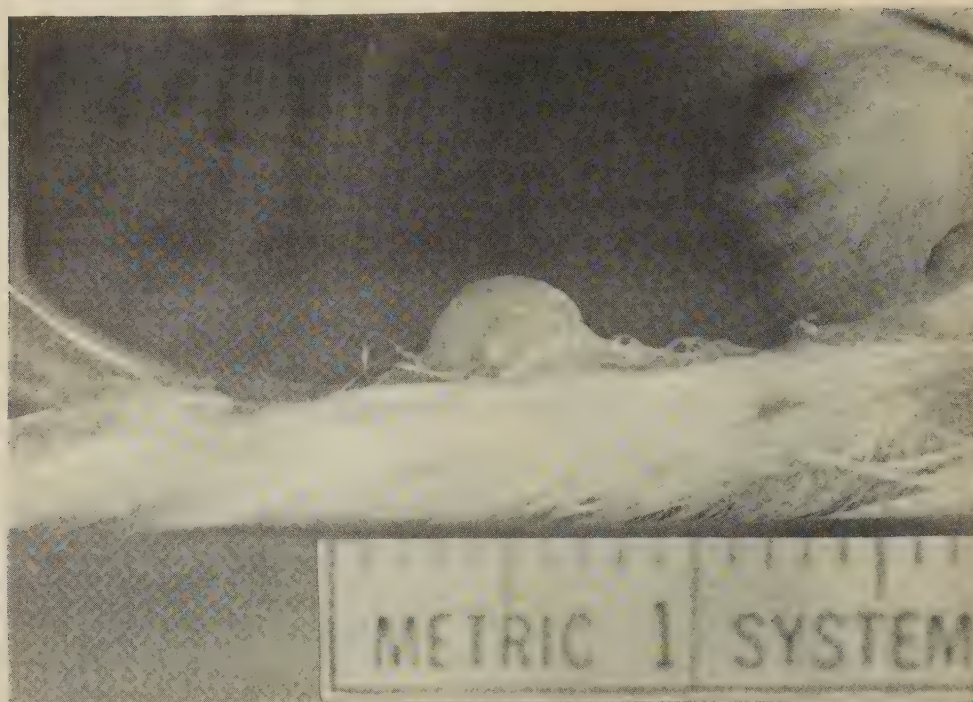


FIG. 4. Side view of altered tumor shown in Fig. 3.

than tumors in control birds.

Fig. 5 shows that when daily injections of hydrocortisone were discontinued almost all altered tumors promptly reverted to typical invasive tumors. In this experiment, 2 groups of 34 chicks each were injected daily with 500 μ g of hydrocortisone and diluent, respectively, beginning 2 days before inoculation of RSV diluted 10^{-2} . All tumors in the treated group appeared as altered tumors and all control tumors were typical and invasive. On 10th

day after inoculation of RSV the treated group was divided in half and injections of hydrocortisone were discontinued in one subgroup and continued in the other. It is clear (Fig. 5) that all but one of the altered tumors promptly reverted to typical invasive tumors in the subgroup in which hydrocortisone was discontinued and the tumors remained altered in those birds which continued to be injected with hydrocortisone. The single bird with an altered tumor which failed to revert when

TABLE II. Modification of Tumor-Response to RSV by Hydrocortisone.

Begun on day:	Daily intramusc. inj. of hydrocortisone Daily dosage, μ g	No. of chicks	Type of tumor:		Latent period LP ₅₀ , days
			Typical, %	Altered, %	
-2	Diluent	30	100	0	4.3
"	20	"	100	0	4.2
"	100	"	90	10	5.3
"	500	"	0	100	6.7
"	Diluent	35	100	0	4.6
+2	500	"	100	0	4.5
0	"	"	97	3	5.4
-2	"	"	0	100	7.6

LP₅₀ = Time in days to produce tumors in 50% of birds.

-2 = 2 days before RSV; +2 = 2 days after RSV.

RSV diluted 10^{-2} .

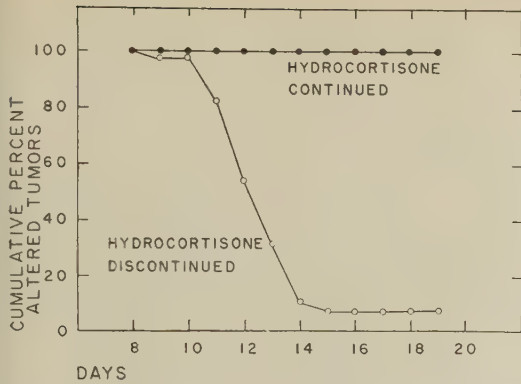


FIG. 5. Reversion of altered tumors after discontinuance of hydrocortisone.

hydrocortisone was discontinued, was also unusual in that the latent period of the altered tumor was unusually prolonged (10 days) by hydrocortisone.

In the single experiment described below the virus content of tumors altered by hydrocortisone was compared with that of control tumors. Groups of 30 chicks each were injected daily with 500 μ g of hydrocortisone and diluent alone beginning 2 days before inoculation of RSV diluted 10^{-1} . Eleven days after inoculation of RSV, 6 birds from the control group and 11 from the treated groups were sacrificed and their tumors were removed aseptically, pooled, and stored in glass sealed tubes at -70°C . Potencies of the 2 pools of tumor tissue were compared with one another and with standard virus as previously described(9). The data indicated that the virus content of altered tumor tissue was lower than that of the control (typical) tumor tissue by a factor of approximately 10.

Discussion. Tumor-response to RSV was strikingly different in chicks that were pretreated with hydrocortisone. This modifying effect of hydrocortisone contrasts sharply with the increased rate of tumor growth observed when injections of hydrocortisone were

begun after inoculation of RSV. Alteration of tumor-response to RSV by pretreatment of chicks with hydrocortisone is markedly affected by dosage of hydrocortisone employed. Thus, altered tumors were produced in chicks pretreated with large doses of hydrocortisone (10 mg/kg) while pretreatment with low doses (0.4 mg/kg) of hydrocortisone not only did not produce altered tumors but the typical, invasive tumors which arose were substantially larger than the control tumors. Studies on the mechanisms involved in these paradoxical effects are in progress.

Summary. When RSV was inoculated into chicks pretreated with large doses of hydrocortisone the tumors appeared later and were greatly altered in appearance. These altered tumors were sharply circumscribed, firm, whitish and grew more slowly than control tumors. When hydrocortisone was discontinued the altered tumors promptly reverted to typically soft and invasive tumors. When lower doses of hydrocortisone were employed or when injections of hydrocortisone were begun after inoculation of RSV the tumors which appeared were typically invasive and grew more rapidly than the controls.

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Acceptance of Tumor Homografts by Mice Injected with Antiserum. I. Activity of Serum Fractions.*† (22185)

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With certain combinations of transplantable tumors and inbred strains of mice, a normally resistant host can be induced to accept a tumor homograft if it is pretreated with preparations of mouse tissues prior to tumor grafting(1-3). Tumor homografts will also survive in mice injected with *antisera* to mouse tissues, produced in either rabbits or mice(4-6).

Since the effect of these antisera is opposite to that expected from a passive transfer of antibody, namely, *inhibition* of tumor growth, the study to be reported in the present paper was conducted to determine whether the sera had the chemical characteristics usually associated with antibodies, or whether they resembled the active substances in the tissues used to induce homograft acceptance. To this end, the sera were subjected to salt fractionation or zone electrophoresis, and the various fractions were tested for their ability to effect the survival of tumor homografts. The effects of heat and of sodium periodate on the activity of the sera were also determined, since it had been shown(2,3,7) that such treatments will destroy the ability of tissue preparations to abrogate host resistance.

Materials and methods. Antisera were produced in rabbits or in C57BL/6Ks (C57 Black) mice. The rabbits were immunized with saline homogenates of freshly secured spleens from strain A mice. The first injection, which was made subcutaneously, consisted of 250 mg of spleen in 1 ml of Freund's adjuvant, containing 3.3 mg of heat-killed BCG. One week later, a series of intraperitoneal injections with saline homogenates of

spleen (100 mg/ml) was started. Injections were given every third day, for a total of 5 injections. The amounts per injection were, respectively: 1.0, 1.5, 2.0, 2.0, and 2.5 ml. Bleeding for serum was done from the ear artery, by a method described by Cohen(8). The sera were stored in the deep-freeze, until needed. These sera, and those produced in mice (described below), exhibited hemagglutinating activity when tested with red blood cells of strain A mice.

Mice were immunized with an extract of a transplantable tumor (Sarcoma I), indigenous to strain A mice. The extract was prepared by homogenizing previously frozen tumor with an approximately equal weight of sterile 0.85% NaCl in a high speed blender. The homogenate was centrifuged at 8500 x g for one-half hour, at room temperature. The supernatant was then diluted 1:10 with sterile 0.85% NaCl, and the diluted material was used for immunization. The diluted supernatant was stored at -26°C. The mice received a series of 5 intraperitoneal injections, 0.5 ml per injection, over a period of 2½ weeks. Twelve days after the last injection, the mice were bled by incision of the lateral tail vein and the serum was separated from the blood clot. In addition to these methods, the antiserum used for zone electrophoresis was produced in mice (C57BL/6Ks strain) inoculated subcutaneously with a tumor homograft (Sarcoma I). The mice were bled from the lateral tail vein 10 days after inoculation. The serum was separated from the clotted blood, freeze-dried in ampoules, and sealed *in vacuo*. Zone electrophoresis was carried out for us by Dr. Peter Bernfeld,† by a method described by Bernfeld and Nisselbaum(9). Five globulin fractions, thus prepared and freeze-dried, were reconstituted by

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TABLE I. Survival of Tumor Homografts in C57BL/6Ks Mice Receiving Prior Injections of Antiserum Fractions Precipitated by Ammonium Sulphate.

(NH ₄) ₂ SO ₄ conc., %	Fraction injected Material ppt.	Amt inj. per mouse	No. of mice dying with tumors*	
Antiserums produced in rabbits				
50-75	Albumin	25 mg	3/5	
"	"	15	0/5	
"	"	5	0/5	
33-50	α, β -globulin	5	5/5	
0-33	γ -globulin	5	5/5	
"	"	3	4/5	
"	"	1	5/5	
—	Unfractionated serum	.5 ml	6/6	
Antiserums produced in C57BL/6Ks mice			Exp. 1	Exp. 2
50-75	Albumin	10 mg	0/5	1/ 5
33-50	α, β -globulin	5	4/5	5/ 5
"	"	2	—	5/ 5
"	"	1	1/5	—
"	"	.5	—	4/ 5
0-33	γ -globulin	5	5/5	—
"	"	2	4/5	10/10
"	"	.5	1/5	3/ 5
—	Unfractionated serum	1 ml	15/15	

* Numerators give the No. of mice dying with tumors; denominators, the total No. of mice in each group.

us with sterile 0.85% NaCl and dialyzed against 0.85% NaCl in autoclaved Visking cellulose sacks for 18 hours, at 4°C, to remove excess salt (since the fractions had been freeze-dried from a large volume of solution). The contents of the sacks were removed and diluted with sterile 0.85% NaCl to the concentration desired for injection into the mice. Salt fractionation of the serum proteins was carried out with a saturated solution of ammonium sulfate which was added dropwise to 20 to 30 ml of serum, maintained at a low temperature in an ice bath. The proteins precipitating in the salt concentrations of 0-33, 33-50, and 50-75% saturation respectively, were collected. Each fraction was then dissolved in distilled water, reprecipitated at least twice more, dialyzed against distilled water in a refrigerator, and freeze-dried. For injection, the freeze-dried material was reconstituted with sterile 0.85% NaCl to the desired concentrations. Treatment with sodium periodate was carried out on the globulin fractions of an antiserum that had been produced in mice. The globulins had been prepared by salt fractionation, as described above. The globulins, in a final concentration of 3.9 mg/ml, were mixed with NaIO₄ dissolved in 0.85%

NaCl, and buffered with phosphate to pH 7.0. The final concentration of NaIO₄ was either 0.01 M, or 0.001 M, and the final concentration of buffer was 0.03 M. The mixtures were kept for one hour at a temperature of approximately 21°C. An equal volume of glucose solution (10 mg/ml) was then added to inactivate the remaining NaIO₄. The measure of biological activity of treated serums and serum fractions was their ability to effect survival of tumor homografts. The various preparations were injected intraperitoneally into the prospective hosts several hours before grafting of the tumor. The transplantable mouse tumor used as the test homograft was the strain A tumor Sarcoma I (SaI). This tumor grows in 100% of A/Ks mice, killing the animals within 3 to 5 weeks after inoculation. Hosts were male mice (except for one experiment in which females were used to test heated serums) of the C57BL/6Ks strain, which is genetically unrelated to the A strain. SaI rarely grows in an untreated C57BL/6Ks mouse. All mice were 2 to 4 months old at the start of the experiments. The grafts were placed subcutaneously, by trocar, in the suprascapular region. Growth of the grafts was followed by means of per-

TABLE II. Survival of Tumor Homografts in C57BL/6Ks Mice Receiving Prior Injections of Antiserum Fractions Prepared by Zone Electrophoresis.*

Material inj., 3 mg/mouse		No. of mice dying with tumors
α -globulin	Fraction II	0/5
Intermediate fraction	III	"
β -globulins	IV	"
Main β -globulin	V	1/5
γ -globulin	VI	5/5

* Antiserum prepared in C57BL/6Ks mice.

iodic palpation, until the mice either died with a progressively growing tumor, or remained without a sign of growth for at least 2 months, at which time they were killed and classified as "negative".

Results. The 2 serum fractions precipitated by 0-33% saturated $(\text{NH}_4)_2\text{SO}_4$ (mainly gamma-globulin) and 33-50% saturated $(\text{NH}_4)_2\text{SO}_4$ (mainly alpha and beta-globulin) appeared to be equally active (Table I). The fraction precipitated by 50-75% saturated $(\text{NH}_4)_2\text{SO}_4$ (mainly albumin) was almost completely inactive. That the activity of the globulin fractions was not due to some possible alteration in the proteins during salt fractionation, was demonstrated by the lack of activity of fractions similarly obtained from normal serum, derived from C57BL/6Ks mice.

The fractions obtained by zone electrophoresis can be assumed to have a degree of purity which cannot be achieved by salt fractionation. Fraction VI (practically pure gamma-globulin) was fully active, while a slight activity was displayed by the neighboring fraction V (the main beta-globulin) (Table II). Fractions II, III, and IV, which respectively are alpha-globulin, an intermediate fraction between alpha and beta-globulin, and lastly mostly beta-globulin (actually 2 beta-globulins), did not effect homograft survival. One point of interest is the observation that the homografts in the mice that had received fraction VI showed a marked initial growth (as compared with uninjected controls or with the animals receiving the other fractions). This was followed by a marked regression and then a resumption of growth, with eventual death of the host. The

possible significance of this is considered below in the discussion.

The results obtained with the heated serums (Table III) show that the antisera produced in both rabbits and mice are fully stable when heated to 56°C for one hour. Whole rabbit antiserum heated to 70°C for one hour retained its activity, though there was a slight impairment, as shown by a slower initial growth rate of the tumors. In the case of the antisera produced in mice, heating to 70°C inactivated the whole serum to a large extent, though not completely, but did not impair the activity of the globulin fractions (produced by salt fractionation).

Sodium periodate did not markedly alter the effectiveness of the antiserum (Table III). There was a slight indication of impairment at the higher concentration (0.01 M NaIO_4), in terms of slower growth of the homografts in the mice receiving the serum treated at this level of sodium periodate. This, however, is in contrast to the complete destruction of the activity of tissue treated with NaIO_4 (7). Though there was a lower number of "takes" in the mice receiving the dextrose-serum mixture, this is not considered significant, since only 4 of the 5 mice which received untreated

TABLE III. Survival of Tumor Homografts in C57BL/6Ks Mice Receiving Prior Injections of Heated Antiserum, or Antiserum Treated with Sodium Periodate.

Material inj. and treatment	Amt inj. per mouse	No. fatal /No. inj.†
Antiserum produced in rabbits		
Whole serum, 100°C/20 min.	1 ml	0/ 5‡
70 / 1 hr	1	5/ 5‡
70 / 1	.5	4/ 5
56 / 1	1	14/14§
Antiserum produced in C57BL/6Ks mice		
α , β -globulins,* 70°C/1 hr	2 mg	3/ 5
γ -globulin,* 70 /1	2	5/ 5
Whole serum, 100 /1	1 ml	0/ 5
70 /1	1	2/ 5
56 /1	1	5/ 5
.01 M NaIO_4	3.9 mg	4/ 5
.001M NaIO_4	3.9	5/ 5
Dextrose	3.9	2/ 5
Untreated	1.0 ml	4/ 5
Dextrose	1.0	15/15

* Prepared by fractionation with ammonium sulfate.

† Males used throughout, except as indicated.

‡ Females.

§ 9 females, 5 males.

serum, in this experiment, grew the homografts. Furthermore, in another experiment, in which dextrose was added to the serum in even larger quantity, the effectiveness of the serum was not diminished.

Discussion. These data, together with others to be published later, indicate that the substance in the antisera which is responsible for the survival of the tumor homografts was not introduced by the injection of the tissue extracts used for immunization, but is an antibody component (or components). This conclusion is supported by the observation that the antisera, unlike tissue preparations, were not inactivated by sodium periodate, and that tissue preparations are labile at temperatures above 60°C(2,3), while the sera are comparatively stable at 70°C. The partial loss of activity of the whole sera heated to 70°C, as compared with the full retention of activity of the globulin fractions similarly treated, is in accord with previously reported observations(10) that isolated protein fractions are more stable to heat than the unisolated proteins in whole serum.

Although the results obtained with the fractions produced by zone electrophoresis would indicate that the activity is most likely associated with the gamma-globulin portion, it is possible that at higher dosage levels than those we used, activity might have been demonstrated by some of the fractions other than fraction VI (gamma-globulin). The limitation on the dosages used was determined by the amount of material on hand, and the inaccessibility of the equipment used for fractionating the sera. The possible importance of dosage is emphasized by the comparatively slow rate of growth of the homografts in the mice pretreated with fraction VI. The

lower activity of this material, as compared with the gamma-globulins produced by salt fractionation, may be attributed to a lower potency of the antiserum used for fractionation. This antiserum had been taken from mice bled 10 days after inoculation with the immunizing tumor, and it is known (data to be published) that the peak potency of the antisera is first reached at between 14 to 21 days.

Summary. Antisera to mouse tissues, produced in rabbits or mice, induce acceptance of an otherwise incompatible tumor homograft in mice, when injected into the host prior to grafting of the tumor. Activity of antisera was associated with the globulin fractions, and was most concentrated in the gamma-globulin fraction. The activity was not impaired by treatment with sodium periodate, or by heating to 56°C. It was slightly impaired by heating to 70°C, and was destroyed at 100°C.

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Tissue Weights of the Rat. I. Normal Values Determined By Dissection and Chemical Methods. (22186)

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Change in body weight, or more particularly tissue weights, is an important and physiologically significant datum in many experimental situations. This paper will describe practical methods for obtaining and expressing tissue weights required for this type of study. These procedures result from experience gained in quantitative dissection of some 300 rats (2-3000 tissue weights) in the course of a 4-year study of alterations in tissue metabolism associated with radiation sickness. They are designed to provide a statistically stable base-line for estimating changes in weight of each major tissue component of the body. The weight of such organs as liver, kidneys, spleen, testes, etc., can easily be obtained by direct dissection, and thus require little special discussion. Unfortunately their total weight constitutes only a minor fraction of total body weight and frequently contributes in a trivial way to a total body weight change. The major portion of body weight lies in the musculoskeletal system. Because of extensive interconnection between muscle, skeleton, marrow and connective tissue, this fraction also presents one of the greatest problems in quantitative separation of different components. For purposes of description, we have arbitrarily divided the musculoskeletal system into 2 major parts: (a) total muscle mass, and (b) non-muscle components which are grouped under the general heading of supportive tissue. A more complete description of the various components of these two major parts will be given as we proceed.

The method for determining how much of the total musculoskeletal weight can be ascribed to each of these two categories depends upon the 2 observations that: (a) some 70% of the muscle mass of the rat can be removed from the supportive tissue in about 20 minutes, and (b) there are sufficiently large differences between the chemical compositions of supportive tissue and muscle so that it is

possible, by direct chemical means, to estimate the amount of muscle still adhering to the supportive tissue at the end of the partial dissection. Chief among the chemical differences between these tissues are the differences in per cent water, per cent ash, and the differences in actomyosin, desoxyribonucleic acid and creatine concentrations. This approach has not only the advantages of speed and ease, but also yields such information as the fresh weight (and per cent water) of supportive tissue fractions and the weight of the total marrow mass which is difficult to obtain by any other method. For further discussions this partially dissected sample, consisting on a fresh weight basis of nearly equal weights of muscle and supportive tissue, will be termed the "stripped carcass." The ideal substance to use in estimating the relative weights of muscle and supportive tissue in the stripped carcass, would be one which is constant in all muscle, absent in skeleton and connective tissue, and is easily and accurately determinable. Empirically, the determination of actomyosin or the per cent of ash have been found most useful for this purpose. Where other analyses are also desired on this same sample the ash procedure is the more convenient. This combination dissection-chemical approach was also used in estimating the total amount of marrow in the skeleton. The useful chemical data in this case were the desoxyribonucleic acid and hemin contents.

Chemical methodology. The reagents and general pattern of procedure for determining actomyosin are those described by Benson, Hallaway and Freier(1). Quickly dissect, weigh and freeze the sample for storage. Chop the frozen sample, place in a water cooled Waring blender cup with 3½ ml of Webers solution (0.6M KCl in bicarbonate buffer) per gram of sample, and blend 8 minutes at reduced speed (furnish 40-60 volts AC to the Waring blender motor). Wash into a beaker

TABLE I. Tissue Weights Expressed in Different Ways.

Tissue	As % of body wt	As % of Wc*—		Data of Column 2 expressed on a:	
		Mean	σ	Fat-free basis	Dry, fat-free basis
GI content†	5.85	—	—	—	—
Depot fat‡	7.08	—	—	—	—
Muscle	45.5	52.3	1.5	52.9	42.1
Supportive	7.68	8.82	.52	8.98	19.24
Skin	18.0	20.7	1.6	19.2	26.6
Liver	4.15	4.77	.53	4.96	4.39
Lung	.56	.64	.10	.66	.43
Heart	.289	.332	.030	.345	.238
Kidneys	.76	.87	.15	.89	.69
Testes	.94	1.08	.13	1.13	.45
Spleen	.213	.245	.034	.256	.187
Brain	.550	.632	.046	.614	.300
Stomach	.502	.577	.038	.581	.435
Small intestine	1.93	2.22	.27	2.21	1.53
Large intestine	.89	1.02	.11	1.00	.63
Thymus	.125	.143	.025	.146	.095
Blood§	4.95	5.68	—	6.04	3.88

* Body wt minus wt of the gastrointestinal contents and depot fat.

† Fresh wt of material in gastrointestinal tract (on *ad lib.* diet of Purina Chow).

‡ Largely adipose tissue, though a small amount of miscellaneous tissue (bladder, etc.) was included.

§ Including fluid loss during dissection (by difference). For further details on blood volume and distribution see Caster, Simon, and Armstrong(8).

with an equal volume of 0.6M KCl, add 10 μ l of 10% ATP per gram of stripped carcass, cover, and allow to stand in a refrigerator for 24 hours. Stir vigorously, transfer 20 ml aliquot(s) into centrifuge tube(s), add 20 ml of 0.6M KCl, centrifuge at 600 g for 10 minutes, transfer supernatant and recentrifuge for 15 minutes. Measure the viscosity of the supernatant before and after the addition of 20 μ l of 5% ATP. The viscosity decrease observed for the stripped carcass is compared with that found for a sample of thigh muscle from the same animal, and the final result expressed as the per cent muscle estimated to be present in the stripped carcass (about 50%). The use of the per cent ash in the stripped carcass as a basis for estimation depends upon the fact that muscle contains only $4.58 \pm .12\%$ ash (on a dry, defatted basis) while the total supportive tissue was found in a separate series of dissections to contain $53.6 \pm .7\%$ (on a dry, defatted basis). The fresh weight figures could be used, but by expressing results on a dry-weight basis one eliminates errors due to evaporation loss and minimizes those resulting from physiological changes in the degree of hydration of the connective tissue. From these data it follows

that for mixtures of muscle and supportive tissue the relation between composition and observed ash can be expressed as $X = 4.58 + .4898Y$, where $X = \% \text{ ash observed in the dry, defatted stripped carcass}$, and $Y = \% \text{ supportive tissue in the sample (expressed on a dry, fat-free basis)}$. Desoxyribonucleic acid and hemin were determined in freshly extruded femoral marrow and in aliquots of completely dissected skeleton which had been dried, defatted and powdered in a Wiley mill to pass a 40-mesh screen. The DNA was hydrolyzed and extracted with 5% trichloroacetic acid at 85°C for 30 minutes, and the desoxyribose determined colorimetrically with Dische reagent(2). Since high concentrations of bone mineral interfere with maximal color development, a series of standards were analyzed in the presence of bone mineral so as to allow correction for this factor. For the determination of skeletal hemin, other aliquots were extracted with 0.1N NaOH, and the iron porphyrin determined by the pyridine hemochromogen method of Remington (3). This procedure is somewhat easier and more desirable because no empirical corrections are required.

Manner of expressing tissue weights. In

comparing and discussing the observed tissue weights of different animals, it would be convenient to express these data in relative rather than in absolute terms. The most usual way of doing this is to express tissue weight as a per cent of total body weight. However, the total body weight contains two portions which may vary considerably from rat to rat and may change very rapidly under stress conditions; *i.e.*, the weights of the (a) body fat, and (b) gastrointestinal contents. It was found empirically that much more stable estimates were obtainable when results were expressed on the basis of a body weight corrected for these variables. The symbol, Wc, will hereafter be used to designate body weight minus the weight of body fat and gastrointestinal contents. The rats used in this study were male white rats (Holtzman Co., Madison, Wis.), 110 ± 10 days of age, weighing 338 ± 20 g. They were received at a weight of 250 to 280 g and raised to the final weight on a diet of Purina Chow in a 25°C room. Dissections were carried out under ether anesthesia. The tissue weight data are summarized in Table I.

Procedure. Skin and eviscerate the anesthetized rat, and remove the depot fat from the carcass. Remove the brain, spinal cord, and any extraneous tissue. Weigh the musculoskeletal remainder and remove a sample of thigh muscle for the determination of ash weight. Divide the musculoskeletal system so that one person can strip the muscle from the head and shoulder girdle, while the other person removes the muscle from the other portions. About 70% of the muscle mass can thus be removed from the supportive tissue in 20-25 minutes. After a few dozen dissections this procedure became highly repeatable, yielding a stripped carcass sample which weighed $18.18 \pm .60\%$ of Wc. After weighing, the stripped carcass was either: (a) analyzed for actomyosin or (b) dried and ashed. As described above, either of these procedures provides an estimate of the residual muscle on the sample. The total supportive tissue weight in this sample was $8.82 \pm .52\%$ of Wc (22 rats) on a fresh weight basis.

Components of supportive tissue. A sepa-

TABLE II. Summary of Supportive Tissue Components in the 338 g Rat.

Tissue	Wt in g			
	Fat	H ₂ O	Fat-free dry wt	Fresh wt
Total	1.07	9.37	15.49	25.93
Skeleton*	.83	6.59	12.73	20.15
Connective	.24	2.78	2.76	5.78
Teeth	.00	.11	.43	.54
Marrow	.01	.09	.81	1.17

* Including teeth and marrow.

rate series of total dissections (22 rats) indicated that the weight of the skeleton (with teeth and marrow, but freed of connective tissue) was $6.86 \pm .33\%$ of Wc, and that the teeth weighed 0.184% of Wc. Analysis of these skeletons for DNA and hemin followed by a comparison of these results with the DNA and hemin contents of freshly extruded femoral marrow led to an estimated total marrow weight of 0.40% of Wc. The weight of the connective tissue (present in the stripped carcass but removed during the total dissection of the skeleton) was estimated by difference to be $(8.82 - 6.86 =) 1.96\%$ of Wc. It should be noted that this does not constitute the total connective tissue of the body, but only that closely associated with the skeleton. All of these data are summarized in Table II, together with analytical data on the weights of water and fat present in each. All data are expressed as grams of weight for the average rat in this study (body weight = 337.7 g and Wc = 294.0 g). The composition of connective tissue was obtained by subtracting the composition of its muscle and skeleton components from the observed composition of the stripped carcass. The dry, fat-free skeleton (complete with teeth and marrow) was 4.33% of Wc, and its ash weight was 2.63% of Wc. These figures are in good agreement with those reported by Savchuck and Armstrong(4) for the 304 g rat. Their information on the individual bones may therefore be considered as an extension of Table II.

Muscle mass. Subtracting the weight of the supportive tissue from the original weight of the musculoskeletal system sample, the total weight of muscle mass is obtained. This method of calculation, again, minimizes er-

TABLE III. Weight of Muscle Groups as % of Total Muscle Mass.

Muscle group	Mean	σ
Thigh	19.18	1.25
Legs	4.48	2.34
Abdominal	16.96	2.37
Shoulder	8.43	1.69
Face and head	4.06	1.58
Back	22.44	1.73
Residual	20.62	6.24
Fat	3.81	.44

rors due to evaporation loss during dissection. The components of the total muscle mass were studied in a separate series of dissections. Table III lists the relative weight of the major muscle groups which comprise this total muscle mass. All results are expressed on a fat-free wet basis and the total fat associated with these muscles is given separately at the bottom of the table. The thigh muscles were obtained by making an incision around the dorsal border of the ilium and extending it medially and ventrally along the os coxae, being careful to avoid the psoas major and psoas minor muscles and the quadratus lumborum. Another incision was made around the knee joint, and the muscles between the two incisions were removed. Then the muscles of the leg from the knee joint on down were removed. The muscles of the foot were not taken. These muscles taken from both legs constitute the leg muscles referred to in Table III. A ventral incision was made in the midline and another incision was made dorsally, just lateral to the transverse processes of the abdominal vertebrae and extended as far caudally as the tip of the ilium and as far cranially as the false ribs. The muscles lying between these two incisions were removed. The psoas major, psoas minor, iliacus, and quadratus lumborum were cut from their origins and insertions. All of these muscles taken together were included in the abdominal muscle group. An incision was made around the scapula and the muscles were stripped from the scapula, brachium and antibrachium. These are referred to as the shoulder muscles. The head was severed at the level of the epistropheus bone and the mandible was removed so as to allow access to the muscles of mastication and the buc-

cinator muscle. The superficial muscles of the head and skull were then removed. This group is referred to as the face and head muscles. The ribs were disarticulated and the intercostal muscles removed. To these were added the major portion of the muscle which could be removed from the trunk. This is defined in Table III as the back muscles. There still remained a certain amount of muscle on the skeleton which corresponded roughly with that present in the stripped carcass sample, and it is here referred to as the residual muscle. This is mainly composed of the anterior and lateral vertebral muscles and the serratus posterior muscles which are difficult to remove from the vertebrae, together with the muscles of the manus and the foot. As seen in Table III the thigh muscles constitute one of the largest of these muscle masses and is, on a percentage basis, also one of the most constant. From its ready availability it furnishes one of the most common sources for muscle samples for chemical analysis.

Other tissues. The items in Table I include two miscellaneous classifications: "body fat" and "blood." The subcutaneous and circumrenal fat constitute by far the largest part of "body fat," but also included in this category are such small tissues as, bladder, pancreas, spinal cord, eye balls, etc. Obviously any of these tissues could be removed from this miscellaneous classification and measured separately if one so desired. The item termed "blood" is even less well defined. At the end of the dissection it is quite usual to find that the sum of the recorded tissue weights equals 94-96% of the weight of the anesthetized animal as measured prior to dissection. By visual inspection it is quite obvious that a certain amount of blood and other fluids have been lost in the course of the dissection. The best evidence(5-8) indicates that some $\frac{2}{3}$ of the blood volume is present in the large vessels and can be lost in the course of this procedure. Since the magnitude of this estimate corresponds so well with the observed 4-6% weight loss, we have arbitrarily termed it "blood." In a series of 22 normal rat dissections, a -0.83 correlation ($P < .01$) was observed between variations in "blood" weight

and the variations in muscle mass weight when expressed as a per cent of Wc. We take this to indicate that another factor entering into this "blood" item is the loss of muscle water by evaporation during the course of the 20-25 minutes required to remove the major portion of the muscle mass. The constancy of the "blood" weight is therefore of interest as an index of the repeatability of the dissection procedure.

Results. Each figure in Table I represents the mean of 16 ± 5 independent dissections. In the first column, these data are expressed as a per cent of the total body weight of the intact rat. As indicated above, a more repeatable and usable result can be obtained if tissue weights are expressed as a per cent of Wc (body weight minus the weights of the contents of the gastrointestinal tract and the depot fat) as is done in the second column. In the third column are given the corresponding standard deviations. These help to indicate the magnitude of change in tissue weight resulting from stress conditions that one might expect to detect with confidence by this dissection-chemical method. When tissue weight comparisons are made on a completely fat-free and fat-free dry-weight basis the results seen in columns 4 and 5 are obtained.

Summary. A combined dissection and chemical approach to the problem of determining tissue weights has been described. It

allows the exact determination of total muscle mass and supportive tissue weight in a fraction of the time required by the complete dissection approach. It also provides estimates of the total marrow weight and the fresh weight of the connective tissue, which are difficult to obtain in any other manner.

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Tissue Weights of the Rat. II. Changes Following 700 r Total Body X-Irradiation. (22187)

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In the first 4-6 days following total body x-irradiation with an LD₅₀ dose of x-ray, the rat loses some 15% of its body weight. An exact description of this weight loss in terms of the weight changes of the individual tissues of the body is of considerable importance in providing a basis for any metabolic study of the nature of radiation damage.

Methods. Groups of 300-350 g male white rats (Holtzman Co., Madison, Wis.) were given 700 r of total body x-irradiation (140 kv, ¼ mm Cu, 1 mm Al) and sacrificed at one of a series of periods following irradiation. Unirradiated control animals for each of the different experimental periods were distributed within each group of experimental ani-

TABLE I. Tissue Weights following Total Body X-Irradiation with 700 r (Expressed as a % of Control(1)).

Tissue	Periods post x-ray								
	3 hr	12 hr	1 day	2 days	3 days	4 days	6 days	8 days	10 days
Muscle	96.7†	94.4†	96.2†	91.3†	88.3†	80.2†	85.3†	85.4†	87.6†
Supportive	99.2	103.1	103.4	113.1†	100.7	113.9†	105.8*	102.4	106.7*
Skin	97.0	97.4	101.4	93.8	86.4	91.1	87.7	94.3	98.2
Liver	101.3	95.8	96.4	90.8	97.2	94.7	94.6	96.1	106.5
Lung	110.8	90.2	101.9	99.4	99.4	112.6	97.0	94.7	97.3
Heart	101.5	105.4	104.8	103.0	97.3	98.8	98.8	97.3	106.6
Kidneys	106.2	104.6	99.0	100.9	86.4	92.1	98.5	94.2	103.3
Testes	99.7	115.0	105.6	108.3	103.1	107.3	88.4	90.2	101.5
Spleen	79.6†	64.2†	57.3†	47.5†	37.4†	36.7†	40.9†	43.1†	60.7†
Brain	103.8	101.6	105.7	107.4*	99.1	106.8*	104.0	97.2	102.1
Stomach	97.1	110.7*	107.5	103.0	106.4	101.1	104.4	86.4†	105.3
Small intestine	98.0	88.7*	83.9†	72.6†	90.1	121.0†	111.2*	86.4*	105.5
Large "	87.4	82.7*	90.0	86.5*	105.1	120.6*	116.0*	97.5	97.8
Thymus	101.8	86.4	42.2†	11.5†	22.2†	22.9†	23.5†	26.7†	43.4†
Depot fat†	106.7	117.3	95.6	74.2	69.7	75.3	87.7	90.6	89.7
GI contents†									
Chow	108.1	100.5	108.5	82.8	75.2	67.2	60.1	90.0	103.7
Synthetic	101.4	143.9	101.0	82.2	120.4	79.6	67.5	45.9	80.7

* Significant at 5% level ($.05 \geq P > .01$).† " " 1% " ($P \leq .01$).

‡ See text for discussion of significance.

mals in a manner designed to prevent inter-group variations from introducing a consistent bias into any portion of the data. Both the normal control data and the procedures for determining tissue weights have been discussed previously(1). All tissue weights are expressed as a per cent of Wc, the body weight minus the weights of the gastro-intestinal contents and body fat(1), and the post-irradiation tissue weights are then expressed as a per cent of the corresponding control tissue weights. This method of expressing the data overcomes artifacts resulting from the large changes in gastrointestinal content weights observed in the post-irradiation period(2). The actual calculation can best be demonstrated by using an example. The average control weight of the heart (after the blood had been expressed from the chambers) was $0.332 \pm .030\%$ of Wc(1). The weight of the heart of rat #88 (body wt = 350.2 g pre x-ray and 316.0 g at dissection) at 2 days post x-ray was 1.024 g. The average control body fat and gastrointestinal contents weights were 15.8 and 19.7 g, respectively, for this experiment. Thus, for rat #88, the heart was

$$\frac{1.024 \times 100}{350.2 - 15.8 - 19.7} = 0.325\% \text{ of control Wc.}$$

This figure averaged with similar data from 6

other rats yielded a mean of 0.342% of control Wc, which, as seen in Table I, was 103.0% of the normal control figure and did not differ significantly from it. The data in Table I represent the averages of over 1600 tissue weights from 193 different rats.

Results. The details of the tissue weight loss at each period following total body irradiation are shown in Table I. Each post-irradiation tissue value represents the mean of 7 ± 2 independent dissections, while the control values are the mean of 16 ± 5 independent determinations(1). The corresponding total body weights, expressed as a % of the pre-irradiation value, are 98.9, 98.2, 98.6, 92.2, 88.2, 87.0, 87.9, 90.5, and 94.5% respectively for 3 hrs, 12 hrs, and 1, 2, 3, 4, 6, 8, and 10 days following x-irradiation.

The loss of body weight following total body x-irradiation is primarily associated with a loss of muscle mass. The skeletal muscle is the only major tissue in which there is a prompt, progressive, and large loss of weight. The losses in liver and body fat weight are small and delayed. This pattern of loss constitutes a major difference between the weight loss characteristic of radiation and that seen in starvation(2). The tissues to lose the greatest amount of weight, on a rela-

tive basis, are the spleen and thymus. The thymus, in fact, comes so close to disappearing at 1 and 2 days following irradiation that its exact dissection and weighing become matters of considerable uncertainty. The total initial weight of both the spleen and thymus is so small, however, that this change has little significance in terms of body weight loss. The changes in the weight of the skin, kidneys and the liver roughly parallel that observed for total body weight. Thus, at each period these tissues represent a nearly constant per cent of the body weight at that time. The gastrointestinal tract shows alternate periods of increase and decrease in weight. The periods of decreased weight correspond roughly with the early and late irradiation death periods.

There are significant increases in the weight of supportive tissue following total body x-irradiation. This is due to the increased hydration of the connective tissue, rather than to any change in the weight of the mineral mass. Complete dissections demonstrated that there was no significant alteration in the ash, dry, or fresh weights of the skeleton or teeth at any of these periods. From the potassium and deoxyribonucleic acid data of Fig. 1, there appears to be a rapid loss of about 30% of the active marrow following x-irradiation. The near equivalence of these two curves indicates that a major part of the skeletal potassium is associated with marrow rather than bone mineral. The other curves in Fig. 1 show the increase in iron porphyrin content and then fat, associated with this destruction of marrow.

The weight of body fat(1) decreases significantly after the 2nd or 3rd day following irradiation. However, most of the body tissues, when carefully dissected, show no significant decrease in their fat content at any time, and marrow (Fig. 1) shows an increase. Thus, this decrease is confined to and characteristic of gross depot fat. The figures in Table I for body fat and gastrointestinal contents represent the means of several experiments which differed considerably in absolute terms. The same relative changes were present and statistically significant in each case,

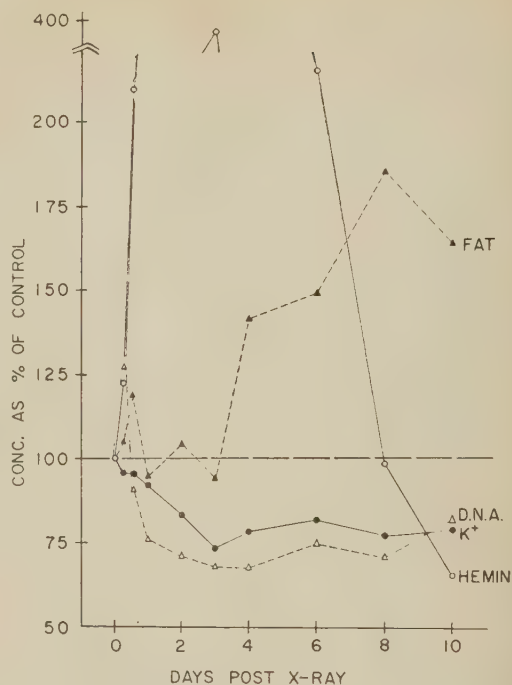


FIG. 1. Changes in composition of skeleton following total body x-irradiation.

however.

Three-hour weight loss. During the process of irradiation the rats were confined, 4 at a time, to a Lucite box whose surface was perforated with 1 cm holes for ventilation. We routinely found a loss of 7.4 ± 4.7 g of body weight during the 0.5-0.8 hr required for the irradiation. The question naturally arises as to whether or not this weight loss is attributable to the irradiation or is a result of the manner in which the animals are handled.

The pertinent data are shown in Fig. 2. Here the per cent weight loss observed under each of 4 different sets of experimental conditions is plotted against time in hours. There was no significant difference between the weight losses observed in the groups of rats that were: (a) confined to the Lucite box for 2 hrs without food and water, (b) left in their cages without food and water for 2 hrs, and (c) sham irradiated, i.e., placed in a carrying cage, transported to the x-ray room, placed into the Lucite box for $\frac{3}{4}$ hr, and returned to the animal room. The weight loss of the irradiated group was significantly ($P = .05$)

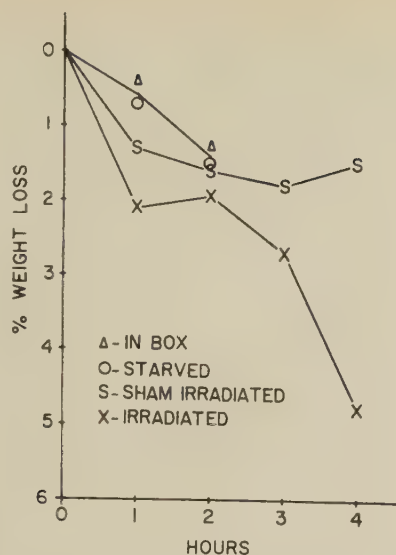


FIG. 2. Loss of body wt resulting from x-irradiation and from 3 control procedures.

greater than that observed for the sham-irradiated group during the first hour. During the next hour the animals were returned to their cages and they ingested some food and water. There was a slight weight gain, but this was promptly reversed, and after 3 hrs the weight of the irradiated group became progressively lower while the sham-irradiated animals recovered lost weight. As seen

in Table I, much of the weight loss of the irradiated animal, even at this early period, can be explained in terms of a loss of muscle mass.

Summary. The weight changes observed in 15 tissues at 9 different periods following total body irradiation with an LD₅₀ dose (700 r) of x-ray have been described and statistically evaluated. The loss of muscle mass explains the major portion of the body weight loss which is seen in the rat during the first 4-6 days following irradiation. Though the percentage weight loss was greater in spleen, thymus and marrow, these tissues are too small to have an appreciable effect upon total body weight change.

The authors wish to thank Judith Poncelet and Ada B. Simon for their technical assistance, and Drs. K. W. Stenstrom and J. F. Marvin of the Department of Radiology for x-irradiating the rats used in this study. This work was carried out under Contract AT(11-1)-106 between the University of Minnesota and the Atomic Energy Commission.

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Vit. B₁₂ Distribution, Determined by Surface Body Counting Following Parenteral Administration of Co⁶⁰ Vit. B₁₂* (22188)

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In a preliminary report it was stated(1) that following intramuscular injection of 1 μg of Co⁶⁰ vit. B₁₂ maximal radioactivity was detected over the liver, spleen and left kidney, in the order named. Activity persisted over the hepatic area during periods of observation in all cases, and ranged up to 315 days. More recently, Glass and his co-workers reported

studies in subjects who received Co⁶⁰ vit. B₁₂ parenterally(2). Their results showed essentially the same pattern of distribution as those previously described from our laboratory.

The present report is a detailed description of our findings on a large group of patients, and a comparison of these results with 6 persons receiving Co⁶⁰ Cl₂.

Materials and methods. The first batch of Co⁶⁰ vit. B₁₂ had a specific activity of 0.240

* Co⁶⁰ Vit. B₁₂ and Co⁶⁰ Cl₂ were supplied by Merck and Co., Rahway, N. J.

TABLE I. Distribution of Radioactivity following Intramuscular Injection of 1 μ g of Co⁶⁰ Vit. B₁₂ (Containing 0.045 μ g Co).

Age	Diagnosis	Observation, days	Disappearance from site of inj., hr			Radioactivity over liver			Radioactivity over spleen			Radioactivity over left kidney		
			Max active, inj., days	Duration of plateau, days	Total activity, days	Max active, inj., days	Duration of plateau, days	Total activity, days	Max active, inj., days	Duration of plateau, days	Total activity, days	Max active, inj., hr	Duration of plateau, days	Total activity, days
47	Normal	315	4	44	315	3	44	315	3	37	166	4	37	166
30	"	98	3	34	98	3	34	98	3	27	98	1	37	55
79	Arteriosclerosis	64	3	63	64	1	63	64	1	56	64	2	3	64
63	Compensated cardiac	145	3	144	145	1	144	145	3	82	122	1	26	89
58	<i>Idem</i>	131	3	101	131	3	101	131	3	54	131	4	34	55
47	Rheumatoid arthritis	155	3	72	155	2	72	155	4 hr	71	141	1	29	64
49	<i>Idem</i>	157	4	156	157	2	156	157	6 "	47	157	3	47	69
52	Cirrhosis of liver	61	3	27	61	2	27	61	1	52	61	1	40	61
64	<i>Idem</i>	182	3	133	182	4 (hr)	133	182	30 min.	29	182	30 min.	15	90
75	P. A. untreated	93	3	92	93	1	92	93	1	78	93	1	16	93
50	<i>Idem</i>	11	3	9	11	2	9	11	4 hr	11	11	4	5	11
61	Gaucher's disease	131	3	130	131	1	130	131	1	116	117	2	45	55
34	Lymphosarcoma	84	4	25	84	2	25	84	4 hr	41	84	4	8	27
45	Polycythemia	80	4	77	80	3	77	80	1	17	80	*	*	*

* Not counted.

TABLE II. Distribution of Radioactivity following Intramuscular Injection of 1 ml of Co⁶⁰ Cl₂ (Containing 0.042 μ g of Co).

Age	Diagnosis	Observation, days	Disappearance from site of inj., days			Radioactivity over liver			Radioactivity over spleen			Radioactivity over left kidney		
			Max active, inj., days	Duration of plateau, hr	Total activity, days	Max active, inj., hr	Duration of plateau, hr	Total activity, days	Max active, inj., hr	Duration of plateau, hr	Total activity, days	Max active, inj., hr	Duration of plateau, hr	Total activity, days
75	Hypertension	77	77	1	12	1	12	20	1	12	5	1	2	4
84	Arteriosclerosis	"	70	2	4	2	4	23	2	4	8	2	2	2
64	Congenital heart disease	"	77	2	24	2	24	55	1	24	20	8	12	2
64	Diabetes mellitus	"	70	1	6	1	6	13	1	24	13	1	6	1
77	P. A. untreated	42	13	2	4	2	4	7	2	3	3	2	4	4 hr
73	Cirrhosis of liver	"	42	2	12	2	12	20	2	4	7	4	4	24 "

$\mu\text{C}/\mu\text{g}$. At a later date this was increased to $0.760 \mu\text{C}/\mu\text{g}$. Each dose was diluted with sterile distilled water so that 1 ml contained $1 \mu\text{g}$ of radiovitamin ($0.045 \mu\text{g Co}/\mu\text{g}$ of vit. B₁₂). The $\text{Co}^{60} \text{Cl}_2$ contained $0.042 \mu\text{g}$ of Co^{60}/ml solution and had a specific activity of $1.86 \mu\text{C}/\mu\text{g}$. All administered doses were 1 ml in volume and were injected into the right deltoid muscle, using a 1-inch 22 gauge needle. A well-shielded sodium iodine crystal scintillation counter having an aperture of 2 cm was placed over 1) site of injection (deltoid muscle), 2) precordium, 3) lower right chest (liver), 4) lower left chest (spleen), 5) left lumbar region (left kidney), 6) lower end of sacrum, 7) lateral aspect of thigh. Radioactivity was recorded for 5-minute periods over each site within $\frac{1}{2}$, 1, 2, 3, 4, 8, 12, and 24 hours after injection. Thereafter, measurements were made at 2, 3, 5, and 7 day intervals for periods ranging from 11 to 315 days. Background counts were subtracted from observed values. No correction was made for Co^{60} decay, since even up to 315 days the correction for physical decay would amount to less than 15%. All but 4 patients were inmates of a chronic disease hospital and received the same diets unless precluded by specific therapy.

Results. Fourteen subjects received $1 \mu\text{g}$ of Co^{60} vit. B₁₂ intramuscularly as described above. The results are tabulated in Table I. Since no significant level of radioactivity was observed over the precordium, sacrum and thighs, these sites were excluded from this table. Activity was always detectable over the liver, spleen and left kidney within 30 minutes after injection, so that the first column under each organ records time after injection when maximal number of counts was noted. In general the highest radioactivity was detectable over the liver, with less activity over the spleen and left kidney in the order named.

Six subjects received 1 ml of $\text{Co}^{60} \text{Cl}_2$ (containing $0.042 \mu\text{g Co}^{60}$) intramuscularly. These data are shown in Table II. The pattern of absorption from the site of injection and distribution over the liver, spleen and

kidney as measured by surface body counting are entirely different from those following Co^{60} vit. B₁₂ administration.

From the above results it is apparent that with the wide variations in time required for achievement of maximal radioactivity after injection, duration of plateau and persistence of activity over the organs named, no clear-cut pattern was observed in healthy or diseased state. Age of subject did not appear to be a factor either. The results in 2 untreated cases of pernicious anemia, one of whom was in moderately severe relapse, were most surprising, since radioactivity was detectable over the liver for as long as individuals were studied.

Since previous animal studies have shown that following parenteral administration of Co^{60} vit. B₁₂ to rats, radiovitamin is found in the liver, spleen and kidney after 3 to 4 days (3,4), it is probable that the radioactivity measured in the present studies represent the B₁₂ complex with Co^{60} still attached. The results with $\text{Co}^{60} \text{Cl}_2$ help to confirm this conclusion.

Conclusions. 1. Following intramuscular administration of $1 \mu\text{g}$ of Co^{60} vit. B₁₂ to normal persons and patients with various medical disorders, radioactivity disappeared from site of injection in 3 to 4 hours. 2. Maximal radioactivity was detected over the liver, spleen and left kidney regardless of clinical condition or age of subject. The wide variations preclude any typical pattern for normal or diseased states. Previous animal studies with Co^{60} vit. B₁₂ and present investigations with $\text{Co}^{60} \text{Cl}_2$ suggest that the measured radioactivity represents vit. B₁₂ complex in organs named.

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Poliomyelitis Antibodies in Inhabitants of the Society Islands,
French Oceania. (22189)

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In 1950, a study had been planned of the distribution of poliomyelitis serum-antibodies among inhabitants of the Society Islands, French Oceania. Unfortunately, the first serum samples obtained were lost in transit to the United States. Then, in March 1951, an unexpected outbreak of the disease occurred in Tahiti, largest of the islands. Collections of new specimens from this and other islands, therefore, had to be conducted during and after the epidemic period. This was the first poliomyelitis epidemic reported from the region, although several sporadic cases of the disease were known to have occurred prior to 1951(1,2).

The accompanying map (Fig. 1) shows the relative position of the Society Islands and distances between them. There is daily boat service between Tahiti and Moorea, while only weekly service operates between Tahiti, Huahine, Raiatea and Bora-Bora. Irregular service takes place in smaller boats by special arrangement between Bora-Bora and Maupiti

—perhaps every 2 weeks or only once a month. Maiao is not in direct line of travel between Tahiti and the larger islands and, because of its small population and inadequate pass, boats do not call there more than 3 or 4 times a year, and always by special arrangement from Tahiti. Tahiti, where the epidemic began, reported 109 cases of the disease, and the other Society Islands, only 2 cases: one from Huahine and another from Raiatea. No paralytic cases were observed in Maiao, Maupiti or Moorea. The high paralytic rate of 360 per 100000 (1,2) was recorded during this epidemic.

The object of this communication is to present results of attempts to isolate virus from stool specimens collected from several frank cases of the disease and to measure complement-fixing and neutralizing antibodies in serum specimens from Tahitian patients as well as from the general populations of Maiao, Maupiti, Moorea and Huahine.

Materials and methods. Stool specimens



FIG. 1.

TABLE I. Schedule of Collection and Testing of Serum Specimens from the Society Islands.

Island	Total No. of sera collected		No. of specimens tested			
			Polio patients		Others	
	Polio patients	Others	C.F.	S.N.	C.F.	S.N.
Tahiti	10	31	8	6	31	6
Maiao		21			20	12
Maupiti		71			46	12
Moorea		76			55	12
Huahine		72			65	15
Totals	10	271	8	6	217	57

were secured from 7 sick patients in Tahiti and all were processed for virus isolation.* A total of 281 serum samples were collected from 5 islands (Tahiti, Maiao, Maupiti, Moorea, and Huahine). Because of breakage upon arrival at the laboratory, only 217 of these were tested for Type 2 antibodies by the C.F. method. Of these, a great number were found anticomplementary, and data reported below pertains to 131 individuals. In addition, 57 specimens were tested for all 3 types of antibodies by the serum-neutralization method (Table I). *Complement-fixation*.† The technic used was essentially the Kolmer-Boerner test(4) which utilizes overnight icebox incubation. Inactivated serum specimens were diluted in normal saline in serial 2-fold dilutions from 1:2 to 1:16. Duplicates of the 1:2 and 1:4 dilutions were also made. Addition of 2 units of Type 2 antigen to the complete line of tubes and of a corresponding volume of saline to the duplicates followed. After distribution of 2 full units of complement, incubation was carried out at 4°C for almost 18 hours. Two per cent sheep cells and 2 units of hemolysin were then added, and the tubes were read after further incubation for 15 minutes at 37°C. The antigen used was derived from acetone-extracted material from suckling hamster brains infected with the MEF₁ strain of Type 2 virus. *Serum Neutralization*.† This was done by mixing equal amounts of inactivated, undi-

luted serum with each of 3 tissue culture suspensions containing about 200 tissue culture doses of Type 1, 2, or 3 virus, giving final concentrations of 1:2 serum and 100 infective doses of virus. In a few cases, where the amount of serum available was limited, initial 1:2 dilutions were used in the mixtures. Adequate normal serum control mixtures were also prepared. All mixtures were incubated in water bath at 37°C for one hour before inoculation into monolayer, monkey kidney culture tubes—3 or 4 per mixture—in medium 199(5). Virus titrations were performed simultaneously. The tubes were observed for cytopathic action for 4 days, at which time the cells of control tubes were completely destroyed.

Results. Of the 7 stool specimens tested, only one yielded poliomyelitis virus of Type 1. This was from the same patient also found to be positive by Dr. Horstmann(3) with whom each of the specimens had been divided.

Results of neutralization tests against all 3 types of poliomyelitis virus on sera collected from individuals on the islands of Maiao, Maupiti and Huahine; and on sera from paralytic cases in Tahiti, obtained from one to 5 weeks after onset of illness, are shown in Fig. 2. It must be remembered that Maiao (column 1) has the least communication with Tahiti and the other Society Islands, whereas Maupiti (column 2) has slightly more, and Moorea and Huahine (columns 3 and 4) are visited by ships at daily or weekly intervals.

Since Maiao remained isolated early in the epidemic, it may be assumed that the neutralization pattern for sera from this island is representative of that which prevailed in all of the Society Islands prior to the 1951 epidemic. Types 1 and 2 viruses were neutralized by less than 30% of the serum samples from Maiao while Type 3 virus was neutralized by 75% of the samples. In addition, analysis of the Maiao results by age groups reveals a total absence of Type 1 neutralizing antibodies in individuals below 25 years of age.

Tahiti presented a different picture, with 93% of the sera from recognized cases of poliomyelitis collected during the epidemic,

* Courtesy of Dr. Charles F. Pait and Dr. Pentti Kokko, Virus Laboratory, Los Angeles County Hospital.

† These tests were performed by the Research Division, Lederle Laboratories, Pearl River, N. Y.

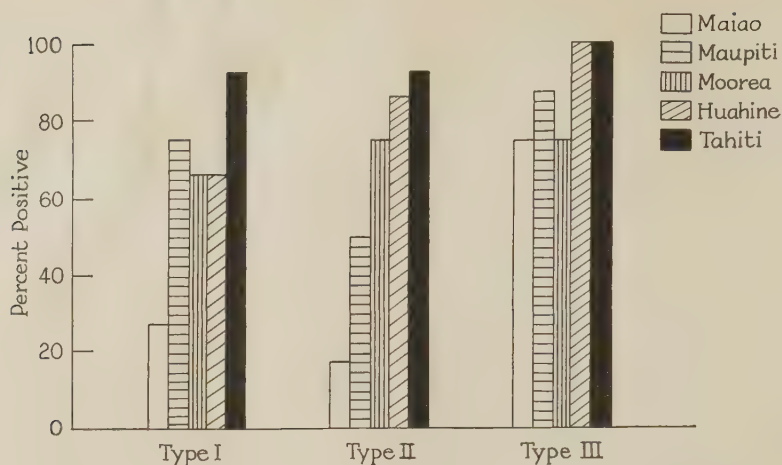


FIG. 2. Types I, II and III neutralizing antibodies in Society Islands.

neutralizing Types 1 and 2 viruses, and 100% neutralizing Type 3 virus. As might have been expected, specimens from other islands showed patterns intermediate between those found for Maiao and Tahiti.

The isolation of Type 1 virus from the stool specimen of one of the patients with paralytic poliomyelitis suggests that this virus was active at the time and that an increase of antibodies to Type 1 virus was to be expected during or after the epidemic. That this occurred is indicated by the fact that 93% of the blood samples collected from post-paralytic cases showed neutralizing antibodies to Type 1 virus, the highest rate in any group tested. It is of interest that the rate of Type 2 neutralizing antibodies was also high in the same group of specimens—93%. In addition, when these sera were tested for complement-fixing antibodies, 75% were found positive for Type 2 virus.

Distribution of neutralizing antibodies by age groups for the 3 types of virus, and of complement-fixing antibodies for Type 2 among sera tested, is depicted in Fig. 3. Specimens from active cases of poliomyelitis infection were excluded from this Figure. It will be observed that, except for the youngest age group, 0-4 years of age, the percentage of Type 3 neutralizing antibodies was higher than of Type 1 or 2; and that the patterns of neutralizing antibodies for Types 1 and 2 were similar to each other.

It is of interest that, shortly after the epidemic of poliomyelitis in Tahiti, Type 2 complement-fixing antibodies were present in normal sera collected from all islands surveyed. Since Casals and others(6) have shown that an increase of complement-fixing antibodies to Type 2 virus may be stimulated by the heterologous Type 1, it is possible that the presence of complement-fixing antibodies to Type 2 virus in the Society Islands at this time was stimulated by Type 1 virus which was active in these islands.

Summary and conclusions. Early in 1951, an epidemic of poliomyelitis occurred in Tahiti during which Type 1 virus was recovered from feces of a paralytic case. Tests on 131 serum samples collected from inhabitants of Tahiti and others of the Society Islands, during and after the outbreak, yielded the following results: 1. A higher percentage of Type 3 than of Types 1 and 2 neutralizing antibodies was found in sera from persons living in the outer Society Islands. On the other hand, higher percentages of neutralizing antibodies against Type 1 and 2 viruses were present in sera of residents of Tahiti during and after paralytic attacks of poliomyelitis than in normal serums from the outer islands. 2. After the epidemic, complement-fixing antibodies for Type 2 virus were present in sera of individuals from the outer islands, and in even higher percentages in sera of post-paralytic patients in Tahiti. Since complement-

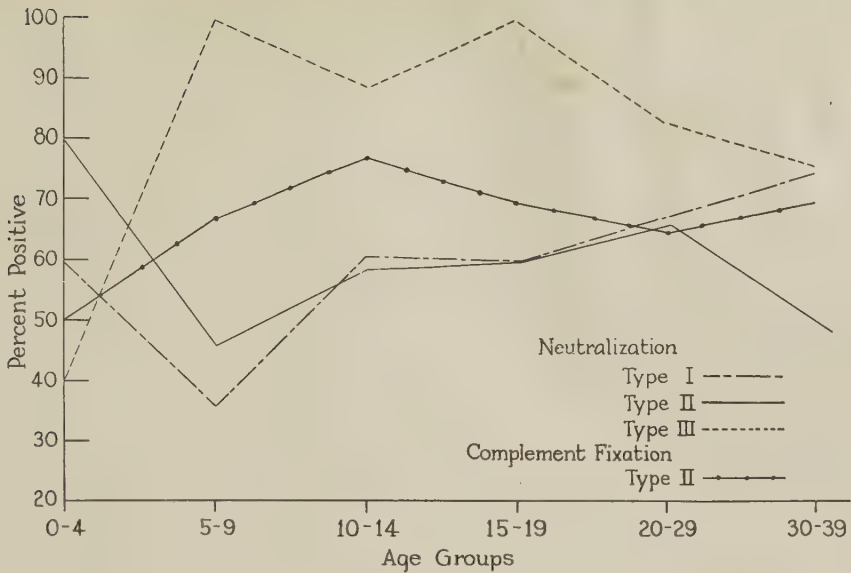


FIG. 3. Neutralizing antibodies by age groups for Types I, II and III and complement fixing antibodies for Type II virus.

fixing antibodies to Type 2 virus may indicate serologic response to the heterologous Type 1 virus, widespread dissemination of Type 1 virus probably occurred at this time. 3. These observations suggest that immunity to poliomyelitis in the Society Islands prior to the epidemic of 1951 was greater to Type 3 virus, and that the outbreak resulted from recent introduction of Type 1 virus, a new type for the area. It is possible that Type 2 virus was present coincidentally.

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Modification of Radiation Injury in the Rabbit. (22190)

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Heterologous tissue transplants or cell suspensions of hematopoietic origin have been reported by Lorenz and his associates(1,2) and Jacobson *et al.*(3) to have a significant effect on survival and hematopoietic regeneration of irradiated rodents. Jacobson first described evidence that suggested the effectiveness of mouse spleen transplants for has-

tening recovery of the blood-forming tissue of irradiated rabbits(4). Congdon and Lorenz (5) reported that rat bone marrow and rat bone had a significant effect on survival of irradiated mice and that guinea pig marrow likewise afforded a significant increase in survival of irradiated mice. Jacobson and co-workers(3) and more recently Cole *et al.*(6)

corroborated the work of Congdon and Lorenz on effectiveness of rat marrow on survival of irradiated mice, but Loutit(7) has been unable to confirm these positive results with heterospecific material.

In earlier experiments, Jacobson did not observe the effect of mouse hematopoietic tissue transplants on survival of irradiated rabbits(4). Hence, the investigation described in this paper was undertaken to determine whether hematopoietic cells from mice enhance survival of X-irradiated rabbits.

Materials and methods. Swift's snuffle-free, young adult rabbits of both sexes, weighing 1.5 to 2.5 kg, were used as recipient animals. The cells used for postirradiation injection were obtained from tissues of the CF No. 1 mouse embryo or from baby mouse spleens or livers. The donor mice were killed by cervical fracture. Embryo tissue was obtained by making a median incision in the abdominal cavity of the pregnant mouse and removing and plunging the entire embryonic sac into cold Locke's solution. The liver was immediately excised from the embryo and suspended in Locke's solution. A homogeneous cell suspension was produced by drawing the combination into a 2-cc hypodermic syringe and expelling it repeatedly. Before cell counts were made, the suspension was drawn into a syringe and expelled through a 26-gauge needle. No appreciable cell clumping was found. The number of nucleated cells per cu mm was determined by making a dilution with 2% acetic acid in a white blood-counting pipette and counting the cells on a standard hemacytometer. Cell suspensions were diluted with Locke's solution so that each 10 cc contained the required number of cells for each rabbit. All injections were given within a few minutes after final dilutions had been made and were administered intravenously into the marginal ear vein of rabbit with a 24-gauge needle within a few hours (0 to 4) after X irradiation.

Cells from spleen, liver, and other tissues of the mouse were obtained in similar fashion. Cells obtained from baby mouse liver or spleen were not contaminated with other mouse tissue cells.

TABLE I. Outline of Experiment to Study Hematologic Changes in Irradiated Rabbits Treated with Cell Suspensions of Mouse Tissue.

Mouse tissue	Total No. cells ($\times 10^6$)	Rabbits (No.)	X-ray exposure (r)	30-day sur- vivors (No.)
None	None	3	None	3
1-day liver	250	4	900	2
1- to 4-day spleen	209	2	"	1
16-day em- bryo liver	240-255	6	"	5
None	None	4	"	1

Rabbits were exposed to 900 r total-body X radiation* before administration of the cell suspensions. Irradiated rabbits that were injected with cell suspensions from nonhematopoietic tissue and nonirradiated rabbits that were untreated served as controls. The experiment to study hematologic recovery of irradiated rabbits following injection of cell suspensions obtained from various tissues of the mouse is outlined in Table I. Table II gives a detailed outline of experiments devoted to a study of survival. As indicated in Table I hematologic studies were made on a small number of animals. Only one control irradiated animal survived beyond the 14th day. Leucocyte, hematocrit, and reticulocyte values were determined for irradiated rabbits that had been injected with mouse embryo liver or with baby liver or spleen; for irradiated, untreated rabbits; and for rabbits that were normal, non-irradiated, untreated controls (Table I). Standard hematologic techniques were employed. X-rays were administered as a horizontal beam to 2 animals at a time. Measurements were made in air with a Victoreen condenser r-meter equipped with a 100-r chamber at the position occupied by center of the 2 cages. At the midpoint of total exposure, the animals were reversed from top to bottom and from side to side. X-rays were generated by a 250-kvp Maxitron machine operating at 30 ma. A 1.0-mm Al and 0.5-mm Cu filter were used. The half-value layer in copper of the filtered beam was 1.43 mm. Exposure rate averaged 44 r per minute at a

* The 30-day X-ray LD50 for these animals in this laboratory is about 800 r.

TABLE II. Effect of Cell Suspensions of Mouse Tissue on Survival of Rabbits Exposed to 900 r Total-Body X Radiation.

Mouse tissue	Total No. cells ($\times 10^6$)	Rabbits (No.)	Survival			
			28-day		120- to 150-day	
			No.	%	No.	%
Embryo liver	200 to 250	19	11	57.8	10	52.6
1- to 2-day liver	"	10	6	60	6	60
1- to 4-day spleen	25 to 30	8	6	75	6	75
Heart, kidney, lung, placenta	32	9	1	11	1	11
None	X-ray only	50	5	10	4	10

distance of 70 cm.

Results. Survival. As indicated in Table II, cells from the mouse embryo or from baby mouse spleen or liver had an appreciable effect on survival of rabbits that had been exposed previously to 900 r total-body X radiation. Of 8 irradiated rabbits that received from 25 to 30 $\times 10^6$ cells from the spleen of 1- to 4-day-old mice, 6 have survived a period of 150 days, whereas only 1 of 9 has survived 120 days that was injected with cells from nonhematopoietic tissue. All nonsurvivors died within the first 26 days of observation period, and no deaths have occurred since that time. Rabbits that received from 200 to 250 $\times 10^6$ cells from the liver of the mouse embryo or from liver of the 1- to 2-day-old mouse had similar survival. Of 29 thus treated, 17 survived beyond 26 days, and only 1 death has occurred (on day 90) dur-

ing remaining 120-day period of observation. Of 50 untreated irradiated controls, only 4 survived (Table II).

Hematologic studies. Figs. 1 and 2 indicate that injection of 200 to 240 $\times 10^6$ cells from the mouse embryo or baby liver or spleen had no appreciable effect on recovery of leucocyte and hematocrit values of the peripheral blood of the irradiated rabbit. The normal reticulocyte value of the irradiated cell-injected group at 10 days as compared with controls (Fig. 3) may be significant but should not be considered as evidence of an earlier recovery in these animals unless confirmed by histopathologic findings or a further hematologic study in which values are obtained at additional periods.

Discussion. The data from this investigation indicate that cells from the embryonic mouse liver or from baby mouse liver or

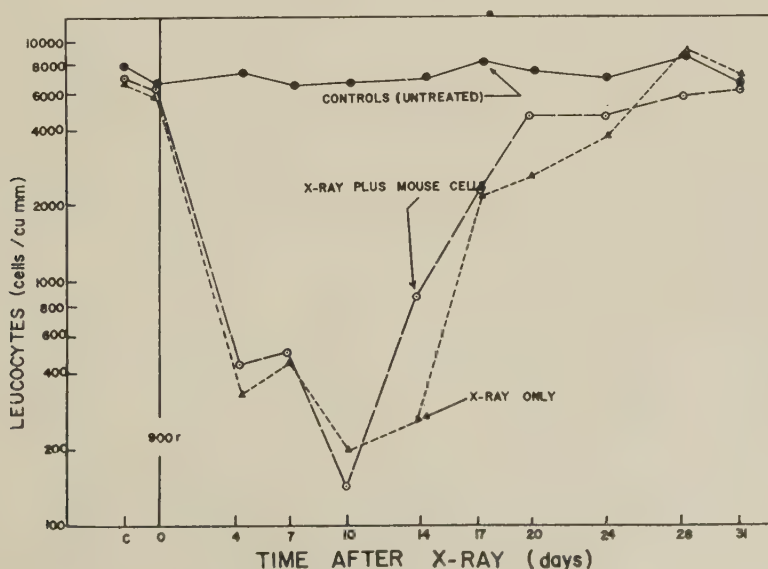


FIG. 1. Effect of cell injections on recovery of irradiated rabbits.

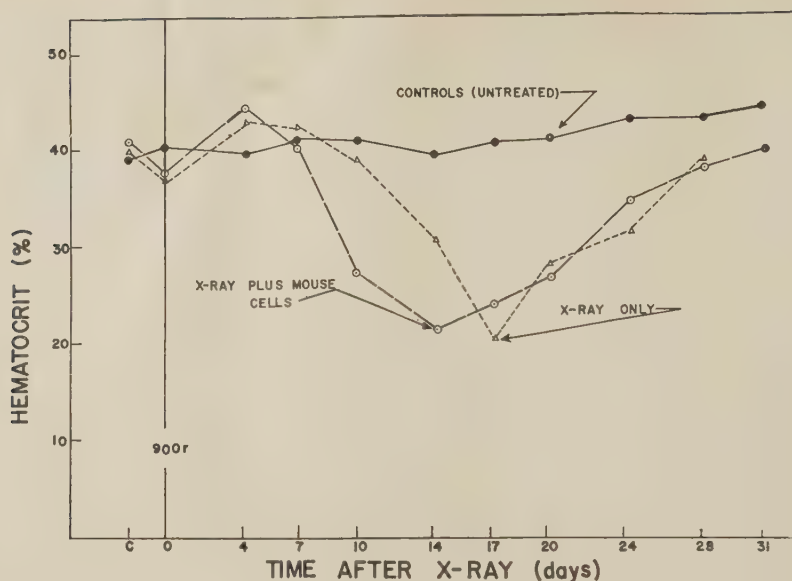


FIG. 2. Effect of cell injections on recovery of irradiated rabbits.

spleen are capable of enhancing the survival of rabbits exposed to 900 r total-body X radiation. These findings supplement those of Lorenz(1,2), Jacobson and his associates (3,4), and Cole *et al.*(6) which show the efficacy of heterologous tissue (rat to mouse, guinea pig to mouse) in prolonging the survival of irradiated mice. Loutit(7), on the contrary, has not been able to increase the

survival of irradiated mice by treating them with heterospecific material. In point of fact, his argument against the benefits of homologous suspensions is based upon the number of late deaths that he observed in irradiated mice that had been treated with cell suspensions made from animals of the same species but of a different strain. It is thus important to emphasize that, in our study, late deaths did not

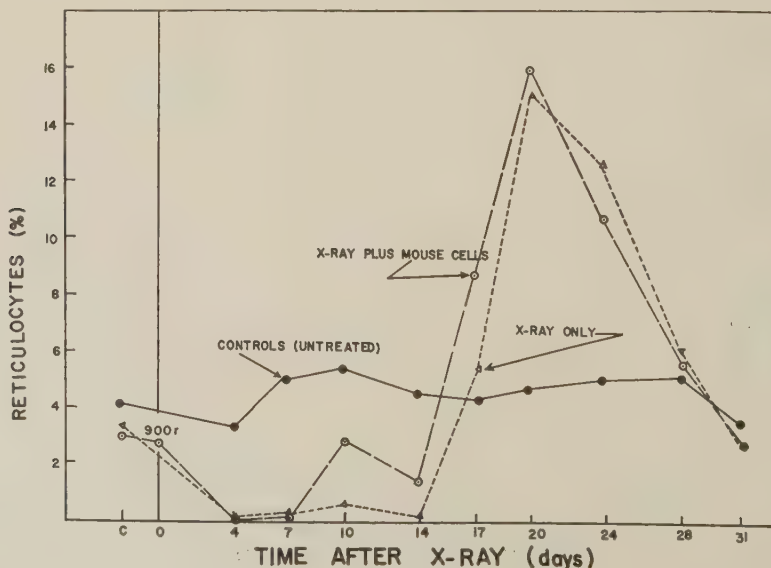


FIG. 3. Effect of cell injections on recovery of irradiated rabbits.

occur in the surviving group of irradiated rabbits that had been treated with mouse tissue.

Previous studies in this laboratory gave evidence that spleen-shielding had only a slight effect on survival of X-irradiated rabbits(8), whereas there was an appreciable effect on survival of mice(4). The possibility that hematopoietic tissue from the mouse is a more potent source of the recovery factor (s) involved in recovery from radiation injury than hematopoietic tissue from the rabbit will require further exploration. Preliminary data in our laboratory suggest that mouse cells from embryonic liver as used in this study are as effective in enhancing survival of irradiated rabbits as are rabbit cells obtained from the liver of 1-day-old rabbits.

The fact that hematologic recovery in the rabbit exposed to 900 r and then treated with cells from the mouse was not appreciably different from that of control rabbits that survived this exposure would seem to indicate that increased survival is not necessarily dependent upon enhanced hematopoietic regeneration. Until histopathologic studies are completed it is perhaps not justified to make such a statement. Evidence of recovery may be present in the bone marrow and elsewhere under conditions of this experiment which are not manifest in peripheral blood.

In some respects, our findings are similar to those from the spleen-shielding splenectomy experiments that have been reported previously(4). Survival of spleen-shielded mice (1025 r) that were splenectomized 1 hour after irradiation spleen-shielding procedure was the same (75%) as that of mice treated similarly, with the exception of splenectomy.

Hematopoietic regeneration began by 3 days and was complete in the nonsplenectomized spleen-shielded mice in 7 to 9 days, while in mice splenectomized 5 minutes after the spleen-shielding-irradiation procedure, regeneration began at 9 days and was complete at 15 days or longer. These observations suggested that increased survival was not necessarily dependent on early regeneration of the blood-forming tissue. Our finding also suggests that early hematologic recovery is not necessary for increased survival.

Conclusions. Postirradiation injection of mouse embryo cells or baby mouse liver or spleen cells enhances significantly the survival of rabbits exposed previously to 900 r total-body X radiation without producing an appreciable effect upon hematologic recovery as observed in the peripheral blood.

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Metabolism of $\text{NaHC}^{14}\text{O}_3$: Effects of Anesthesia and Route of Dosage.* (22191)

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A number of workers(1,2) have studied health physics aspects of $\text{NaHC}^{14}\text{O}_3$ metabolism and others(3,4) have shown bicarbonate carbon to be fixed in a variety of metabolites. We were unable to find adequate information in the literature on early times of the time course of C^{14}O_2 excretion or of effects of anesthesia upon this excretion. The effect of a further variable, route of dosage, also does not appear to have been reported. For adequate interpretation of the fate of labeled metabolites that are oxidized, it is necessary to know the fate of C^{14}O_2 so produced. In a following study important use is made of the time course of C^{14}O_2 excretion following acetate dosage.

Methods. Rats were given tracer $\text{NaHC}^{14}\text{O}_3$ under a variety of conditions and respired C^{14}O_2 and C^{12}O_2 were measured. Following multiple dosage of the $\text{HC}^{14}\text{O}_3^-$ the rats were sacrificed and tissue lipides analyzed for C^{14} activity. Sprague-Dawley, adult, male, 200-g white rats trained to feeding as before(5) were used exactly one hour post-prandially. Following injection, the rats were placed in the metabolism chamber of the apparatus(6) and respiratory C^{14}O_2 activity data collected from the decimal scaler component. Occasional one-minute CO_2 samples were collected. The total CO_2 and C^{14}O_2 exhaled was collected and the amount and activity of the CO_2 was determined. Animals anesthetized were given 3.5 mg/100 g body weight of Nembutal. The anesthesia was given 45 minutes after start of the one-hour eating period, which was thus 15 minutes before the tracer bicarbonate was given. Radiobicarbonate was prepared by acidification of Oak Ridge BaCO_3 and absorption of the released CO_2 in NaOH . The pH of the solution was 8.2 and contained 0.38 mg

NaHCO_3/ml with an activity of $10.84 \mu\text{C}/\text{ml}$; 0.4 ml was the usual dose. Aliquots of the standard NaHCO_3 solution and respiratory CO_2 samples collected in alkali were directly plated(7) and counted under a thin end window tube as before(8). Because of low activities found in tissue samples, the fatty acids were directly plated, the cholesterol was plated as the digitonide and liver glycogen was also directly plated. Counting rates were corrected to "infinite thickness" by the application of appropriate factors previously determined in this laboratory. Specific activity figures listed below are on the order of one-tenth of the specific activities obtainable at "infinite thinness." A total of 9 experiments was conducted on 2 rats. Since each animal was subjected to the 3 conditions studied, "internal control" of experimental conditions was maintained. Intravenous injections were made in the saphenous vein at the junction of saphenous and lateral plantar veins. This route of injection permits a rapid yet quantitative administration of label. Following intravenous injection of HCO_3^- the animal was put into the metabolism system with dispatch because of the rapid rate of C^{14}O_2 excretion. The greater part of the bicarbonate dose having been exhaled in 3 hours allowed us to reuse the rats 24 to 48 hours after previous dosage. Forty-eight hours following the second dose of bicar-

TABLE I. Dosage Schedule for the Two Rats Receiving Tracer Bicarbonate.

Exp.	Animal No.	Dose, ml	Route	Anesthesia
1	V5-1	.4	I.P.	—
2	2	"	"	+
3	3	"	"	—
4	4	"	I.V.	+
5	V6-1	.8	I.P.	—
6	2	.4	"	+
7	3	"	"	—
8	4	"	"	—
9	5	"	I.V.	+

* This investigation was supported largely by contract No. AT(45-1)-225 of the U. S. Atomic Energy Commission.

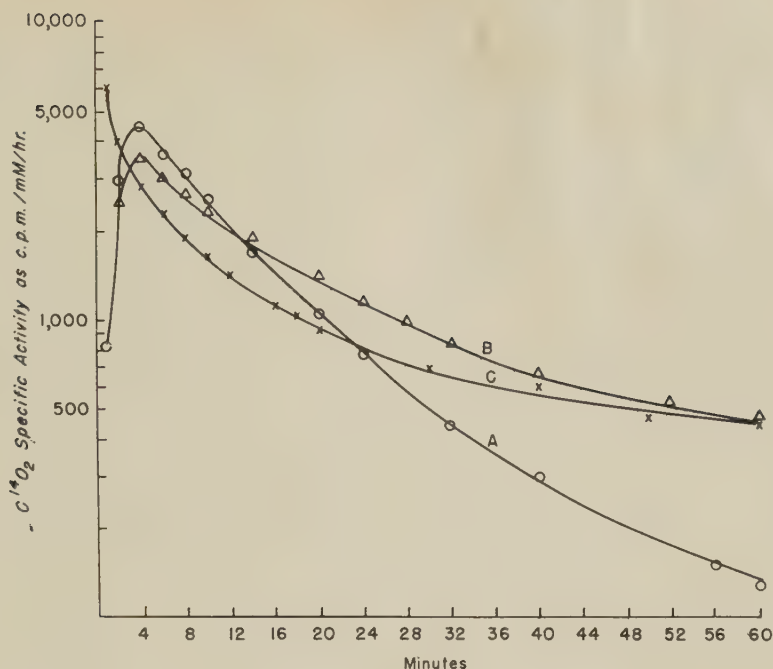


FIG. 1. Respiratory C^{14}O_2 activities following $\text{NaHC}^{14}\text{O}_3$ dosage. Curve A, avg of 4 experiments with 2 rats, 0.4 ml NaHCO_3 intraperitoneally. Curve B, as with the experiments of A but with rats anesthetized with Nembutal. Curve C, avg of 2 experiments in which anesthetized animals were given the tracer $\text{NaHC}^{14}\text{O}_3$ intravenously.

bonate the animals were placed in the metabolism chamber and respiratory C^{14}O_2 activity monitored. No activity above background was detected.

Results. Table I presents the details of the nine experiments from which time-course CO_2 data were obtained. Exp. 1 and 3 represent duplicate intraperitoneal, nonanesthetized runs on the first animal and Exp. 7 and 8 similar duplicate runs on second animal, giving a total of 4 trials of these conditions. Exp. 2 and 4 tested the effects of Nembutal upon C^{14}O_2 excretion after intraperitoneal dosage. Runs 4 and 9 tested the combined effects of Nembutal and intravenous dosage. The effect of dosage size was tested in Exp. 1 and 5.

The c.p.m. obtained from the decimal scaler (6), were converted to specific activities by dividing the actual count by the mM CO_2 produced per hour. This latter figure was obtained from the amount of CO_2 collected during the entire experiment. In each experiment 2, one-minute samples of CO_2 were col-

lected and analyzed separately. The amount of CO_2 produced per minute measured by these samples agreed, within the usual range of such values, with the average CO_2 figures obtained from the total CO_2 collection.

The specific activity values used as the ordinate of Fig. 1 represent c.p.m. per mM CO_2 produced per hour, the counts used, being those recorded by the gas flow counting of the system described. These counting rates can be converted to equivalent infinitely thick BaCO_3 counting rates by multiplying by a factor of 5.3.

In Fig. 1 are presented the 3 curves that represent average results obtained from experiments detailed in Table I. Curve A represents the time-course of respired C^{14}O_2 following intraperitoneal dosage without anesthesia and curve B the effect of Nembutal anesthesia upon the C^{14}O_2 excretion. Following the intraperitoneal dosage both with and without anesthesia, C^{14}O_2 specific activities quickly increased and reached a maximum in about 6 minutes, demonstrating the rapidity

of peritoneal absorption processes. Attainment of similar maxima at similar times suggests that the intraperitoneally administered Nembutal did not greatly affect HCO_3^- absorption from the peritoneal space. The C^{14}O_2 specific activities decreased following

the maxima but plots of $\log \text{s.a.}$, $\log \frac{1}{\text{s.a.}}$, or $\log \frac{1}{\text{s.a.}^2}$ fail to reveal a straight line function

for this decay, indicating the complex nature of the simultaneous distribution and excretion of the labeled bicarbonate. In the period immediately following the maxima, 7 minutes were required to reduce by $\frac{1}{2}$ the s.a. of the intraperitoneal animals not anesthetized while 11 minutes were required for the anesthetized animals to reduce their C^{14}O_2 specific activities to one-half. Also, the anesthetized animals excreted only some 85% as much of the dose as did the nonanesthetized animals in the first hour. The decreased "turnover" of the HCO_3^- in the anesthetized rats is consistent with the slowing of respiration associated with Nembutal anesthesia. Upon awakening, in the 40- to 60-minute period, the rat treated with Nembutal struggles briefly and hyperventilates, which results in a sharp upward deflection in CO_2 specific activities. Following this, the respiratory rate of the rat slows and approximates that of the non-Nembutalized animal, for the slopes of the CO_2 specific activity curves become similar.

Curve C of Fig. 1 presents the average of the C^{14}O_2 time-course curves of the 2 experiments in which anesthetized animals were given bicarbonate intravenously. C^{14}O_2 appears in the respired air with such rapidity that it is difficult to make an accurate reading of the scaler at one minute. At this early time the respiratory CO_2 specific activity was found to be maximal, as might be expected from the probable maximum s.a. of blood HCO_3^- . The shape of the time-course curve plotted as C is similar in shape to curve B, confirming the effect of Nembutal anesthesia upon CO_2 turnover as seen in the intraperitoneally injected preparations.

Following the collection of CO_2 data in 4 experiments on animal V5 and in

5 experiments on animal V6, the rats were fractionated as previously described(5) and total saponifiable and nonsaponifiable lipid fractions isolated from liver, gut, carcass and skin. Liver glycogen was isolated from V5 and directly plated and counted as were the lipid fractions. The C^{14} activity of the lipides was extremely low and the data is thus not presented in detail. In the cholesterol (nonsaponifiable) fractions, the maximum per cent incorporation found was only 0.004. The maximum incorporation found for fatty acids (saponifiable fraction) was 0.05%. Compared to equivalent dosage with acetate- C^{14} in one hour postabsorptive animals, the cholesterol and fatty acid incorporation was less than 0.005 of that usually found. It is clear that the C^{14}O_2 released from tracer dosage, even from rapidly metabolized labeled substrates such as acetate, does not to a significant degree(4) label body fatty acids or cholesterol.

The liver glycogen of V5 after the 4 doses of tracer bicarbonate contained about 0.01% of the label. This figure has little absolute meaning because of the multiple nature of the dosage and the period of time elapsed during the 4 experiments.

Summary. The time-course of respiratory C^{14}O_2 has been followed after intraperitoneal and intravenous dosage of $\text{NaHC}^{14}\text{O}_3$ into anesthetized and non-anesthetized rats. Nembutal has been shown to affect the turnover of injected bicarbonate through its depression of respiration. The anesthetic did not slow peritoneal absorption. The multiple bicarbonate dosage used did not lead to appreciable labeling in tissue lipids, and the contribution of C^{14}O_2 to secondary labeling reactions can under these conditions be ignored. Liver glycogen "fixed" a small but measurable amount of the C^{14} label.

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Hyperglycemia and Increased Liver Glycogen in Rats After X-Irradiation.*† (22192)

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It is well established that whole-body x-irradiation of rats results in an increase in level of liver glycogen(1-4). On the other hand it has been reported that liver glycogen of rabbits is markedly reduced following x-irradiation(5). Although x-irradiation raises liver glycogen level of rats and decreases that of rabbits, blood sugar levels in both animals are increased by exposure to x-irradiation. Kohn(6) found that in rats irradiated with 600-900 r, plasma glucose level rises within 2 to 3 days and McKee(4) has found, on the first day after irradiation, an increase in blood sugar levels of rats exposed to 1500 r. Steadman and Grimaldi(7) observed a hyperglycemia in rabbits following x-irradiation. This hyperglycemia increased with increasing dose and at every dose (500 to 2000 r) reached a maximum 4 hours after irradiation, thereafter declining to normal levels within 24 hours. Hyperglycemia was completely prevented by insulin. Therefore, these authors postulated that irradiation causes a mobilization of liver glycogen into glucose and that the normal supply of insulin in the rabbit is not sufficient

to prevent hyperglycemia. Since irradiation increases liver glycogen levels in the rat, the question arises as to the reason for hyperglycemia. Before an attempt was made to answer this question, it appeared that a detailed study of the relationship of liver glycogen and blood sugar levels to dose and time after irradiation would be of value. Data on these points are presented in this paper.

Methods. Animals. Sprague-Dawley female rats about 10 weeks of age were used. In each experiment, animals of uniform weight were divided into groups of either 4 or 5 rats each. *Radiation technics.* Radiation source was Westinghouse X-ray machine of 250 KVP and 15 ma, with filter of 0.5 mm copper and 1 mm aluminum. All doses were delivered at 24.5 per minute at a target-to-skin distance of 40 inches. During irradiation rats were restrained in acetate-plastic boxes and slowly rotated under the beam to insure uniform exposure(8). Non-irradiated, control animals were caged in the same manner for the same period of time. *Experimental design.* In Exp. 1 only liver glycogen was determined. In Exp. 2 both liver glycogen and blood sugar levels were determined, whereas in Exp. 3 only blood sugar levels were measured. Rats subjected to whole-body x-irradiation were fasted for 24 hours, exposed to single x-ray dose and the fast continued. Doses administered were 500,

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† The opinions or assertions contained herein are private ones of the authors and are not to be construed as official, or reflecting the views of the Navy Department.

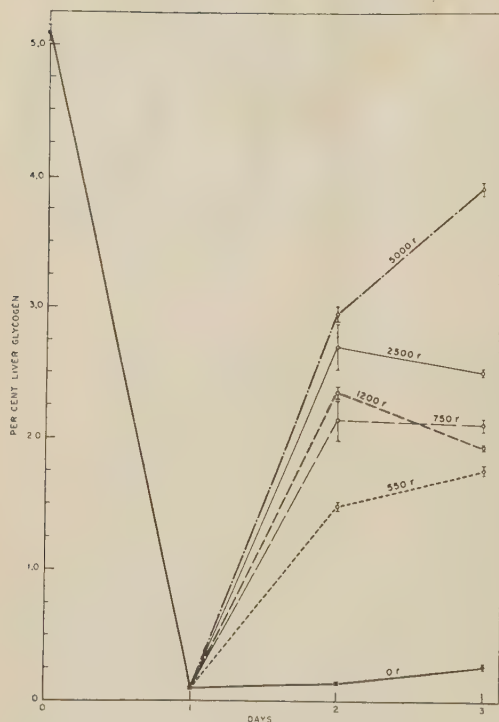


FIG. 1 (Exp. 1). Liver glycogen levels as a function of time in fasted, x-irradiated and fasted, normal rats. Fast was begun on day 0, and rats were exposed to x-radiations on day 1. There were 5 rats in each group of control animals and 4 rats in each group of fasted, x-irradiated animals. Means and stand. errors of means are plotted. Glycogen was determined by method of Good *et al.*(9).

750, 1200, 2500, and 5000 r. Eight rats were used at each dose level. Four rats from each group were sacrificed 24 and 48 hours after x-irradiation. Non-irradiated control animals were sacrificed in groups of 5 after 0, 24, 48, and 72 hours of fasting. *Analyses.* Rats were anesthetized with pentobarbital. Chest cavity was cut open and heart blood was withdrawn into a heparinized syringe. When liver glycogen was determined the liver was removed, blotted free of adhering blood and weighed. A liver sample of appropriate size was obtained, weighed and immediately analyzed for glycogen. In Exp. 1 glycogen was determined by the method of Good *et al.*(9). In subsequent experiments liver glycogen was determined by the method of Kemp(10) and blood sugar level by the method of Mendel *et al.*(11). The results are presented in Fig. 1 to 3.

Results. Liver glycogen levels. It is evident from Fig. 1 and 2 that fasting produces, within the first 24 hours, a sharp drop in percentage of liver glycogen. This drop is followed by a slight increase in liver glycogen during the next 48 hours. Whole-body x-irradiation, on the other hand, produces, in the fasting rat, a sharp rise in liver glycogen within 24 hours after time of exposure. The percentage of glycogen found in livers of irradiated rats increases with increasing dose. In each experiment the glycogen content of livers of rats exposed to 5000 r was considerably greater 48 hours after time of irradiation than at 24 hours. On the other hand, the percentage of glycogen found in livers of rats exposed to the other dose levels was about the same at both 24 and 48 hours after irradiation. At no time during the course of any experiment was the percentage of glycogen in the liver of the fasting, irradiated rat equal

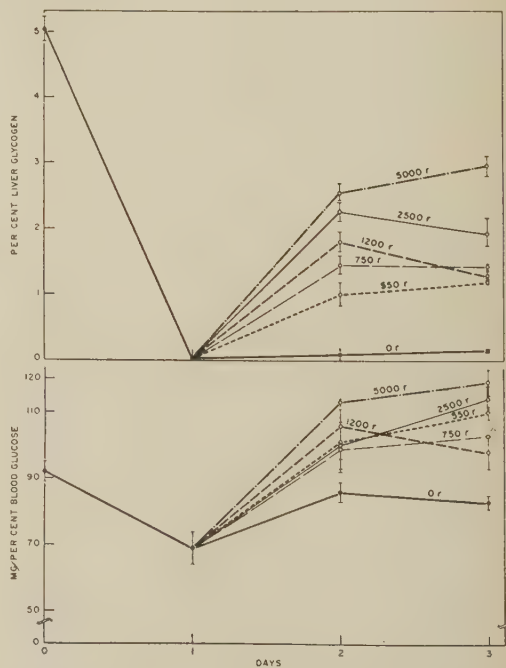


FIG. 2 (Exp. 2). Blood glucose and liver glycogen levels as a function of time in fasted, x-irradiated rats and normal control rats. Fast was begun on day 0 and rats were exposed to x-radiations on day 1. Five rats in each group of controls and 4 rats in each group of fasted, x-irradiated animals. Means and stand. errors of the means are plotted. Liver glycogen was determined by method of Kemp(10).

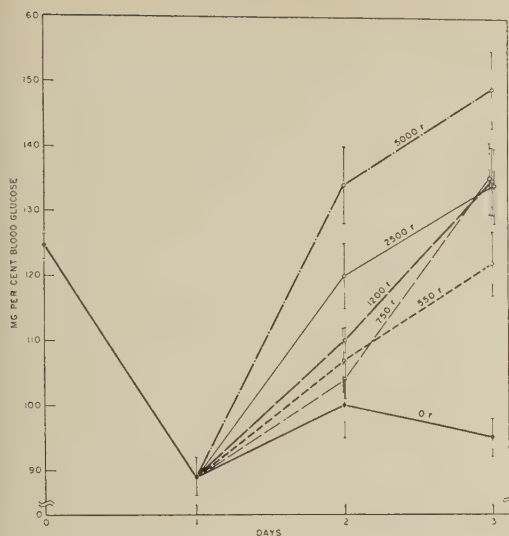


FIG. 3 (Exp. 3). Blood sugar levels as a function of time in control and fasted, x-irradiated rats. Fast was begun on day 0 and rats were exposed to x-radiations on day 1. Five rats were used in each group of controls and 4 rats in each group of fasted, x-irradiated animals. Means and standard errors of means are plotted.

to or greater than that found in the normal, fed animal.

Blood sugar. From Fig. 2 and 3 it can be seen that during a 72-hour fast the blood sugar level drops by about 20% during the first 24 hours, rises slightly during the next 24 hours and remains at this new level over the final 24 hours. On the other hand, within 24 hours after exposure to x-irradiation the blood sugar level of fasting, x-irradiated rat increases and, at every dose (with the exception of 1200 r in Exp. 2), it continues to increase during the next 24 hours. Forty-eight hours after exposure to x-irradiation, concentration of sugar in blood of irradiated rats exceeds that found in normal, fed rats. There appears to be no clear relationship between the x-ray dose and blood sugar level. However, in each experiment the highest blood sugar levels, at both 24 and 48 hours after irradiation, are found in rats exposed to the highest dose (5000 r).

Discussion. The data presented indicates that exposure of fasting rats to whole-body x-irradiation results in an increase in liver glycogen and blood sugar levels. If the increased glycogen levels are a result of in-

creased gluconeogenesis, then it is obvious that x-irradiation must cause appropriate glucose precursors to be made available. That glucose precursors are made available from mobilized protein is indicated by the finding of increased urinary excretion of urea in fasted x-irradiated rat(12). However, it appears that the availability of glucose precursors is not the only factor that determines amount of glycogen found in the liver following irradiation. This is indicated by the fact that an increased dose of x-irradiation over the range of 550 to 5000 r results in increased liver glycogen, while daily excretion of urea, the major end product of protein metabolism, is not altered by increasing the dose over the range of 500 to 2500 r(12). Furthermore, since it has been found that hypophysectomy prevents deposition of glycogen in the liver of irradiated rat(3), it seems that stimulation of the pituitary-adrenal system is necessary for the conversion of glucose precursors to liver glycogen. Since glycogen deposition increases with increasing dose, whereas availability of glucose precursors apparently does not, it seems possible that, once glucose precursors are available, the amount of glycogen deposited in the liver is determined by strength of stimulus delivered to the pituitary-adrenal system.

The fact that the fasted, x-irradiated rat is hyperglycemic clearly shows that x-irradiation does not, as was concluded by McKee(4), merely prevent the fall of blood sugar due to fasting. The hyperglycemia of the x-irradiated rat does not appear to be necessarily related to the accumulation of glycogen in the liver. This is indicated by the fact that, at all doses, blood sugar level of irradiated rat continues to rise 48 hours after irradiation, whereas (with the exception of the rats exposed to 5000 r) maximum levels of liver glycogen are obtained within 24 hours.

Summary. 1. Whole-body x-irradiation increases the percentage of liver glycogen in the fasting rat. At 24 hours after time of irradiation glycogen deposition was shown to increase with an increase in dose. 2. In rats exposed to 5000 r the percentage of liver glycogen was greater 48 hours after the time of irradiation.

tion than at 24 hours. In animals exposed to 550, 750, 1200 or 2500 r the percentage of liver glycogen was not greatly different at 48 hours and 24 hours after the time of irradiation. In no case did the liver glycogen levels in the irradiated rats equal or exceed that found in normal, fed rats. 3. Whole-body x-irradiation increased the blood sugar levels in fasting rats. Larger increases were noted at 48 hours after the time of irradiation than at 24 hours. At 48 hours after the time of irradiation the blood sugar level was, in every case, greater than that of fed, normal rats.

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A Winter Isolation of Western Equine Encephalitis Virus from Hibernating *Culex tarsalis* Coquillett. (22193)

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The means of survival of Western equine encephalomyelitis virus (WEE) during the winter months has been a perplexing question since the disease was first recognized. Several explanations have been proposed for reintroduction or reappearance of the virus every year in certain endemic areas. The 3 most common theories are: 1. Migrating birds bring the virus northward each spring. 2. Arthropods, other than mosquitoes, harbor the virus during winter and infect susceptible birds during the most active stages of the arthropod life cycle. 3. Infected mosquitoes hibernate over the winter and infect susceptible birds and mammals during the following spring. It is the purpose here to present our findings and discuss the last possibility. As pointed out by Eklund(1,2) the conditions under which a mosquito-vertebrate cycle fails to account for maintenance of virus must be better understood before alternative mechanisms can be explored intelligently.

During the summer months WEE virus has been recovered from *Culex tarsalis* Coquillett

frequently and from other mosquitoes occasionally in endemic areas(3-8). Hibernating *C. tarsalis* have not previously been reported in such numbers, and few of these have been tested for the presence of WEE virus. Although winter isolations of WEE virus from this species have been made in California during November and January(9) it is felt that these mosquitoes did not represent true hibernators. We consider the following factors to be indicative of true hibernation: the presence of fat bodies, lack of feeding activity of mosquitoes, absence of male mosquitoes, quiescence of female mosquitoes, and temperatures prohibitive for mosquito survival outside of the hibernating sites.

Methods. Mosquito hibernation sites. *C. tarsalis* mosquitoes were found hibernating in abandoned mines during winter months. These mines were in the foothills in Boulder and Larimer Counties, Colo., between elevations of 5000 and 8000 feet above sea level. The temperature from Dec. 22 through Feb. 20, was between 12.2°-13.3°C in a mine near

TABLE I. Hibernating *Culex tarsalis* Mosquitoes Collected in Colorado during the Winter of 1953-1954.

Date collected	Date tested	No. mosquitoes	No. pools	From	Location
12/15/53	1/19/54	38	1	6 mines	7 miles W of Masonville
30	"	587	12	3 "	3 " E " Jamestown*
"	"	1	1	1 mine	7 " W " Boulder
1/ 7/54	"	612	12	1 "	3 " E " Jamestown
13	2/16	1	1	1 "	1 " NW " Sunshine Canyon
14	22	63	1	Mines in vicinity of Jamestown	
21	16	3	1	" " " Central City	
22	22	41	1	5 coal mines	12 miles NE of Fort Collins
2/ 9	"	15	1	1 mine	2 miles E of Masonville
Total		1361	31		

* Isolation made from one pool of 50 mosquitoes.

Masonville, and relative humidity in this mine fluctuated from 30 to 80%.* However, during the latter part of February, after a long dry period, the relative humidity dropped to 15%. No mosquitoes were found in this mine after this sharp drop. The mine shaft entrances varied from 4 to 6 feet in diameter; the shafts proper extended from 120 feet straight back from the entrances to side shafts which extended into unexplored depths. *Collection of mosquitoes.* From Dec. 15, 1953 to Feb. 9, 1954, mosquitoes (*C. tarsalis*) were collected at random from the abandoned mines. The mosquitoes were resting approximately 10 to 50 feet from mine entrances. In no cases where mine shaft was absolutely dark during daylight hours were any mosquitoes found. A few mosquitoes were found in mines after the date in spring when presence of active adult *C. tarsalis* in the open indicated that they were leaving hibernation sites. Collections were made by locating mosquitoes on rock surfaces with a flashlight and aspirating them with a hand aspirator. The mosquitoes were taken to the laboratory in small cages, identified, then stored at -20°C in 10 x 75 mm tubes until tested for virus. *Processing of mosquitoes.* The mosquitoes were ground by chilled mortar and pestle in inactivated 20% normal horse serum (pH 7.4). A volume of 2 ml of diluent was used for pools of 25 to 75 mosquitoes, and 1 ml for those pools of less than 25 mosquitoes. The suspensions were

centrifuged 10 min. at 1000 x G at room temperature. Three-tenths ml of buffered saline (pH 7.4) containing 2 mg of streptomycin sulfate and 1000 units of penicillin G sodium were added to 1 ml of supernatant materials and the mixtures incubated overnight at 4°C . Each antibiotic-treated supernate was inoculated subcutaneously in 0.03 ml amounts into 4 freshly hatched chicks, which were then observed for a 72-hour period(10). The remainder of supernatant material was ampuled, then frozen in alcohol and dry ice and stored at -70°C for reference.

Results. A total of 1361 female *C. tarsalis* mosquitoes, as shown in Table I, was collected and made into 31 pools. One pool of 50 mosquitoes collected on Dec. 30, 1953, killed 4 out of 4 chicks within 24 hours. None of the other pools killed chicks. The brains of the 4 chicks showed no evidence of bacterial contamination when cultured in thioglycollate medium. Upon passage of the 20% brain suspension, 3 of 4 chicks died in 24 hours with no evidence of bacterial contamination.

The brains of these chicks, as well as the original infected mosquito suspension, were sent in a frozen state to the Communicable Disease Center, Virus and Rickettsia Section, Montgomery, Ala., where the agent was confirmed as WEE virus by complement-fixation tests and serum neutralization tests in mice.

Discussion. The isolation of WEE virus from hibernating *C. tarsalis* mosquitoes collected in December shows this species to be capable of harboring the virus through a period of adverse weather conditions. Al-

* As measured by a recording hygrothermograph manufactured by Instruments Corp. of Baltimore, Md.

though the isolation does not prove that *C. tarsalis* could carry enough virus through an entire winter to infect birds and mammals in the spring, it furnishes the best supportive evidence for this theory that has yet been obtained. The ratio of positive pools to total pools tested of hibernating mosquitoes is not incompatible with the results of summer collecting and testing.

Congregation of *C. tarsalis* in certain mines is noteworthy, and relatively large numbers of mosquitoes found in these mines suggests that temperature and relative humidity therein are tolerable for mosquito hibernation. Although mines are artificial hibernating sites, and it is obvious that the majority of individuals of this widespread species must hibernate in locations other than mines, conditions found in these mines may furnish clues as to types of natural mosquito hibernation sites.

Summary. A total of 1361 hibernating female *C. tarsalis* mosquitoes, comprising 31 pools, was collected in mines in Colorado foothills from Dec. 15, 1953, to Feb. 29, 1954. One of these pools, collected Dec. 30, 1953,

contained WEE virus upon inoculation into freshly hatched chicks. The finding of such large numbers of hibernating *C. tarsalis* and the recovery of WEE virus from them, may contribute to the knowledge of the overwintering mechanism of this virus.

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Recent Isolations of Encephalomyocarditis Virus. (22194)

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Strains of encephalomyocarditis (EMC) virus have been isolated on several occasions. The earliest of these isolations was made by Jungeblut during poliomyelitis investigations, first from cotton rats in 1940 (Columbia-SK strain)(1) and from hamsters in 1943 (MM strain)(2), but these were initially regarded as strains of poliovirus. A virus producing encephalomyocarditis in animals, and thus named encephalomyocarditis (EMC) virus, was isolated in 1945 by Helwig and Schmidt from a chimpanzee dying of myocarditis at Dania, Fla.(3,4). This virus was later found to be immunologically identical to Columbia-SK and MM viruses(5). In 1948 Mengo encephalomyelitis virus was isolated at Entebbe,

Africa, from a rhesus monkey(6) and this virus was similar or related to the EMC virus. Since then similar types of virus have been isolated in Africa from man, mosquitoes, and monkeys(7). The present report describes 3 more isolations of viruses belonging to the EMC group.

On June 12, 1952, specimens of lung, liver, spleen, kidney and stomach contents from a mandrill baboon which had died of unknown cause at the Anthropoid Ape Research Foundation Farm in Dania, Fla., were received at the Communicable Disease Center from the Miami Regional Laboratory of the Florida State Board of Health for study. The baboon had died 24 hours after the first visible signs

of illness. All that was noted by the veterinarian in charge during this 24-hour period was that the baboon could not stand and refused food. The animal had been at the Foundation farm in Florida for 3 or 4 years.

Methods. The liver and spleen were pooled as were kidney and lungs. Ten per cent suspensions of these pools and the stomach contents were prepared and, after addition of suitable amounts of penicillin and streptomycin, each of the 3 suspensions was inoculated intracerebrally into groups of 5 mice. All inoculated mice died between the 4th and 14th days. Cultures of the brains revealed bacterial contamination. These brains were inoculated into a second group of mice which died on 3rd to 6th day. No evidence of bacterial contamination was observed when brains of these mice were cultured on blood agar and in thioglycollate broth. A suspension of 2 of these 2nd mouse passage brains had a mouse intracerebral LD_{50} titer of $10^{6.3}$. The titer of 3rd and 4th mouse passage material was $10^{6.3}$ to $10^{6.8}$.

Results. Neutralization tests were carried out by incubating mixtures of 10-fold dilutions of the agent and undiluted antisera to various viruses at 4°C overnight and then inoculating each of the mixtures intracerebrally into 5 mice. Neutralization was not considered positive unless the LD_{50} endpoint of test serum was 1.5 logs lower than normal serum control. The agent was not neutralized by antisera to eastern or western equine encephalomyelitis or St. Louis encephalitis viruses, but was neutralized by EMC antiserum. Antisera to EMC virus and the new agent were prepared by a single subcutaneous inoculation of guinea pigs with live material. Reciprocal neutralization tests were made with these sera. Both EMC virus and the new agent were neutralized to essentially the same degree by heterologous as well as homologous antiserum.

A second isolation of EMC virus was made from a monkey brain submitted by the Florida State Health Department on Feb. 9, 1953. The virus was also isolated from brain of a mouse which had been inoculated at the Florida State Health Department Laboratory

with brain material from this same monkey. This monkey had been housed at the same farm in Dania, Florida, where the baboon mentioned earlier had been kept. The monkey species and its clinical history were not provided with the specimen and could not be obtained later. A suspension of the brain of this monkey, upon inoculation into mice, yielded a virus which behaved essentially like that isolated earlier from the baboon.

EMC virus was isolated again from a squirrel brain submitted by the Florida State Health Department Laboratory on Feb. 11, 1954. This squirrel, which originated in Palm Beach County, Fla., had appeared sick and bit a child who handled it. Therefore, it was captured to have it examined for evidence of rabies infection. As far as we are aware, this inadvertent finding represents the first isolation of EMC virus from a wild animal indigenous to North America, although antibodies to the EMC group of viruses has been previously demonstrated in wild rats in the southern United States by Warren and co-workers(8).

The second mouse passage material of the baboon strain was titrated in 3-week-old mice by the subcutaneous, intramuscular, intraperitoneal, intranasal, and intracerebral routes. An inoculum of 0.03 ml brain suspension material was used. The agent titered about 10-fold higher by the intracerebral than by the intramuscular, subcutaneous, and intraperitoneal routes, and about 1000-fold higher than by the intranasal route. The incubation periods or symptoms did not vary significantly by the various routes. Mice receiving the lower dilutions died in 3 to 5 days after inoculation, while death from the more dilute suspensions could be delayed up to the 10th day. Those mice receiving the low dilutions of brain suspension became weak, with ruffled hair coats, and they would be found dead the day after these symptoms appeared. Occasionally convulsions would be seen. Mice receiving higher dilutions would first be seen walking with an unsteady gait. This would be followed by a complete paralysis of the hind legs. These symptoms would continue for one to 4 days and then be followed by prostration

and death.

Cotton rats inoculated intranasally or subcutaneously with a 10^{-3} dilution of virus became weak and died within 48 to 72 hours after inoculation, while guinea pigs displayed a febrile response only and survived subcutaneous inoculation.

The lesions in inoculated mice consisted of glial nodules scattered through the corpus callosum, internal capsule, thalamus and mid-brain. There was almost complete destruction of the Purkinje cells of the cerebellum. The cerebral cortex was essentially free of lesions. The lumbar portions of the spinal cord showed an occasional necrotic neuron in the ventral gray horn. Demyelination was present in certain fiber tracts and nerve trunks. The heart showed interstitial round cell infiltration and mild fiber fragmentation in the auricular myocardium. The spleen, lungs, liver, gut,

and kidneys were free of demonstrable lesions.

Summary. During the past 3 years encephalomyocarditis virus was isolated twice from captive primates imported to Florida and once from a wild squirrel originating in Florida.

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Effect of Temperature on Activity of Guinea Pig Complement. (22195)

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When human complement (hu C') and sensitized sheep erythrocytes (EA) interact, both extent of lysis and shape of the lytic curves obtained are markedly dependent on temperature(1). These findings have recently been explained(2) on the basis of proportionality between temperature and rates of formation and decay of an intermediate complex EAhuC'_A.

Since the great majority of both experimental and routine laboratories have used guinea pig (g.p.) rather than hu C' in their investigations, and since the choice of 37°C as reaction temperature appeared to be quite arbitrary, the effect of temperature on lysis by g.p.C' was investigated. These studies were greatly facilitated by data from several laboratories(3,4) on EA_{g.p.C'}1,4,2, the complex between EA and g.p.C'1, C'4 and C'2.

Methods. Sheep blood in 500 ml lots was preserved with the addition of 2000000 units

of penicillin "G" and 25 ml of 0.15 M Trisodium ethylenediamine tetraacetic acid (Na₃E.D.T.A.)(4). Veronal buffer(5) containing 5×10^{-4} M Mg⁺⁺ and 1.5×10^{-4} M Ca⁺⁺ was used for all dilutions and washings. Pooled guinea pig sera, stored in small lots at -25°C, were the source of g.p.C'. Kinetic analyses were carried out according to the technic of Mayer and Levine(6). Bath temperatures were held constant to $\pm 0.1^\circ\text{C}$. All solutions were equilibrated at the temperature of the experiment before mixing.

Results. Two flasks, each containing 10 ml of EA, 10 ml of buffer and 5 ml of g.p.C'/170 were incubated at 37°C, and 27°C respectively. After sampling was completed, 2 more flasks were run at 37°C and 32°C. The reaction curves are plotted in Fig. 1. The extent of reaction at 37°C was considerably less than at either 27°C or 32°C.

The next experiment was designed to study

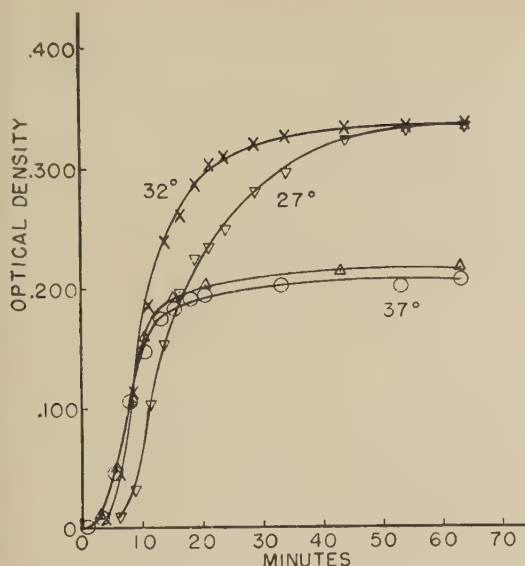


FIG. 1. Effect of temperature on lysis by a constant volume of g.p.C'.

the effect of temperature on formation of the intermediate complex EAg.p.C'1,4,2. The complex was prepared by the method of Leon *et al.*(4), using a sublytic dilution of g.p.C'. Flasks containing 30 ml of cells, 30 ml of buffer and 15 ml of g.p.C'/540 were incubated at 37°C, and 27°C and later the same day at 37°C and 32°C. At intervals 3 ml samples were withdrawn, mixed with 0.5 ml of g.p.C'/

40 and 0.2 ml of 0.15 *M* Na₃E.D.T.A., stoppered and incubated at 32°C for one hour. Na₃E.D.T.A. binds Ca⁺⁺ and Mg⁺⁺ and therefore prevents further reaction of any complement components except for C'3(4,7). The C'3 present in the g.p.C'/40 will lyse only those cells that have reached the stage EAg.p.C'1,4,2, through reaction of EA with g.p.C'/540. The results are plotted in Fig. 2.

Discussion. In the presence of a constant amount of C'3 the extent of lysis is proportional to the quantity of EAg.p.C'1,4,2 available(4,8). The quantity of EAg.p.C'1,4,2 available will, in turn, be a function of the quantity of C'1, C'4 and C'2 present and of the reaction temperature (Fig. 2). Thus, at 27°C and 32°C, where the concentration of EAg.p.C'1,4,2 is maintained at a high level for a considerable length of time, more lysis is obtained than at 37°C where the concentration of intermediate is smaller and its stability lower.

In this laboratory, where both hu C' and g.p.C' are used, the majority of lytic reactions are now carried out at 32°C, the temperature previously shown to be optimal for hu C'(1).

Summary. Guinea pig complement is considerably more reactive at 27°C and 32°C than at 37°C. These findings have been ex-

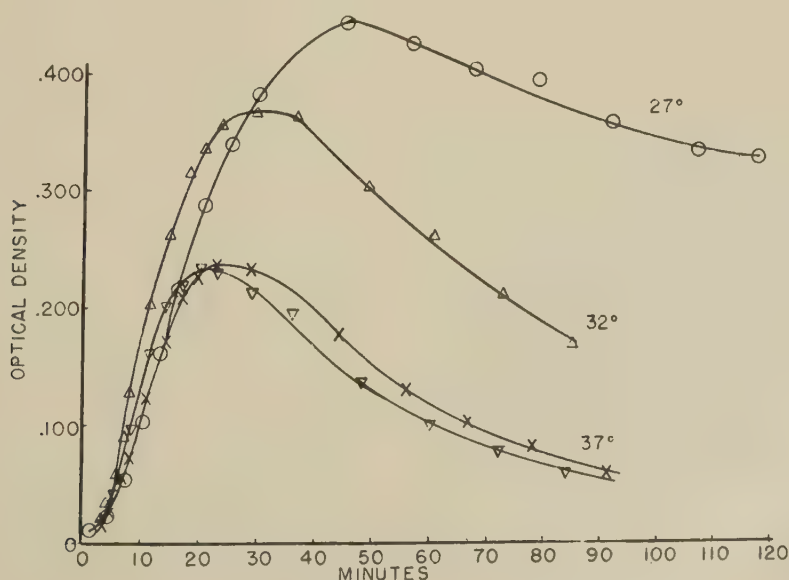


FIG. 2. Effect of temperature on formation and decay of EAg.p.C'1,4,2.

plained by differences in the maximal formation and the stability of an intermediate complex, EAg.p.C'1,4,2.

The author wishes to express his appreciation for the technical assistance of Miss Edna Chapman.

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Effect of X-Irradiation and Hypophysectomy on Globule Leucocytes In Rat Intestine.* (22196)

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Globule leucocytes are distributed widely in urinary(1), respiratory(2) and digestive mucous membranes (Fig. 1) of the rat. They are distinguished by presence in their cytoplasm of abundant, coarse, metachromatic acidophilic inclusions and by the fact that they occur only in the epithelium and tunica propria. Cytological evidence indicates that they arise by direct transformation from lymphocytes(3). Like lymphocytes, they are reduced in number by treatment with corticotropin or cortisone(2). Because of these ob-

servations and the well-known radiosensitivity of lymphocytes, this study was made of the response of globule leucocytes to total body x-irradiation. Observations are reported also on effect of hypophysectomy.

Procedure. Female Sprague-Dawley rats weighing 150-190 g were used. During early experiments, control rats were pair fed against irradiated animals. Since it was observed that reduction in food intake was without effect, the animals were fed *ad libitum* in the final experiments. Diet consisted of Purina Laboratory Chow supplemented twice weekly with greens, oranges, and a vitamin concentrate. Temperature of animal quarters was maintained at $75 \pm 3^\circ\text{F}$. All irradiated animals (except as noted below with relation to shielding experiments) were exposed to whole body irradiation while held in flat cylindrical holder constructed of $\frac{1}{4}$ -inch sheet Lucite and radially divided into 9 equal compartments. Radiation was administered by Keleket Theron X-ray therapy machine operated at 200 KV with $\frac{1}{2}$ mm Cu and 1 mm Al filtration. Distance from center line of x-ray tube to table on which animal container rested was 110 cm. Dose rate was indicated by Victoreen rate meter and was held constant at about 11 r/min by varying x-ray tube cur-

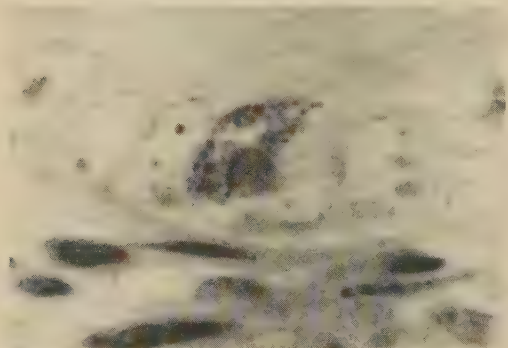


FIG. 1. Globule leucocyte in epithelium of duodenal crypt of hypophysectomized rat. $\times 1450$.

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TABLE I. Effect of X-Irradiation on Globule Leucocytes in the Ileum.

Days after irradiation	No. rats	X-ray dose (r)	Mean No. globule leucocytes/section	P values*
0	20	—	251.3 $\pm$ 104.6†	
1/2	2	650	84.0 $\pm$ 15.3	<.001
3/4	1	"	22.5	
1	4	"	19.2 $\pm$ 8.0	<.001
2	2	125	92.0 $\pm$ 13.5	"
2	3	650	7.8 $\pm$ 1.3	"
4	5	"	2.6 $\pm$ 1.2	"
5	2	"	2.5 $\pm$ 1.0	"
6	2	"	5.3 $\pm$ 2.3	"
7	5	"	6.5 $\pm$ 4.5	"
14	4	"	13.8 $\pm$ 1.2	"
21	2	"	21.5 $\pm$ 11.0	"
30	2	"	71.2 $\pm$ 6.5	"
35	2	"	201.5 $\pm$ 32.5	NS
43	2	"	220.4 $\pm$ 21.6	"
53	2	"	257.8 $\pm$ 8.8	"

NS = Not significant ($P > .05$).

* Counts compared with those for the rats which received no irradiation.

$$\dagger \text{Stand. dev.} = \sqrt{\frac{\sum x^2 - \bar{x}^2}{n}}$$

rent within range of 20-25 ma. Output was calibrated by giving a timed exposure to Victoreen r-meter chamber inserted within animal chamber in a position receiving average radiation intensity. At autopsy, samples of ileum were fixed in Helly's fluid. Paraffin sections were cut at 5 μ and stained in phosphotungstic acid hematoxylin and basic fuchsin. Globule leucocytes were counted in 3 non-adjacent sections from each animal and these counts averaged. The number of animals sacrificed at various time intervals after irradiation and radiation doses employed are summarized in Table I. To determine whether x-irradiation acted directly or indirectly on globule leucocytes, additional animals were divided into 3 groups: a. those having a segment of intestine exteriorized and protected by lead shield during irradiation; b. those treated like group a, but with shield made of paraffin; c. those treated like group a, but without exposure to x-irradiation. Animals were anesthetized with subcutaneous injections of Nembutal (0.04 or more mg/g body weight), and a loop of small intestine was exteriorized through incision in the flank. In initial experiments, 4 rats were irradiated

simultaneously while fastened to plywood square with their ventral sides up and shield toward center of board. They were arranged symmetrically with relation to the x-ray beam. For experiments at 600 r and 800 r, smaller shields were used to permit irradiating anesthetized animals in the regular Lucite holder instead of tying them down as in earlier experiments. The intestinal loop was anchored in depths of the shield by a loose stay suture passed around the intestine (through the mesentery) and around a wire hook at end of shield. The shield itself was fastened to skin of the animal by sutures through wire loops at each end of the shield. These groups were irradiated at 20 r/min by a Pikens X-ray machine operated at 235 KV. In all shielding experiments, one limb of the shielded segment of intestine was marked at the entrance to the shield with a mattress suture with knotted end pointing toward shielded loop. At autopsy, small samples of shielded and non-shielded intestine were taken approximately 1.5 cm on each side of this suture. The procedure for preparation and study of these tissues was the same as for those from non-shielded animals. Experiments on hypophysectomized animals were performed in 8th or 9th post-operative week. In all cases, completeness of operation was established by microscopic examination of serial sections of the pituitary capsule. All incompletely hypophysectomized animals were eliminated.

Observations. In non-hypophysectomized rats there was a statistically significant reduction in number of intestinal globule leucocytes within 12 hours after irradiation, with a 99% disappearance of cells by 4th and 5th days after irradiation with 650 r (Table I). Beginning about the 6th day after irradiation and continuing at a relatively slow rate until about the 30th day, globule leucocytes increased in number. During 4th week after irradiation, reappearance of cells accelerated and by 35 days their number was within the normal range. Since globule leucocytes arise within the tunica propria from small lymphocytes(3), which are radiosensitive, it is pertinent to note that rate of increase in absolute lymphocyte count in the circulation(4)

TABLE II. Effect of X-Irradiation and Shielding on Globule Leucocytes in the Ileum.

Exp.	Type of shield	Dose (r)	Days after irradiation	No. rats	Mean No. globule leucocytes/section	P values
I	Lead	0	1, 4, 7	6	170 $\pm$ 50 *	NS †
		0	1, 4, 7	6	215 $\pm$ 40	" †‡
II	Paraffin	650	2, 4, 7	13	5.6 $\pm$ 2.3	<.001†
		"	2, 4, 7	3	4.4 $\pm$ 4.3	NS ‡
		"	2, 4, 7	6	286.7 $\pm$ 44.4	" †
III	Paraffin	800	3	3	5.9 $\pm$ 3	<.001†
		"	3	1	9.7	
		"	3	2	188.2 $\pm$ 20.9	NS †
IV	Paraffin	"	4	11	1.6 $\pm$ 1.9	<.001†
		"	4	1	4	
		"	4	9	148.4 $\pm$ 42.8	" †
V		0	0	11	109.8 $\pm$ 63.4	" †
Hyp.	Lead	600	4	8	10.9 $\pm$ 9.4	NS §
		"	4	6	100.6 $\pm$ 85.7	"

$$* \text{Stand. dev.} = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n}}$$

† Compared with non-hypophysectomized, non-irradiated rats (Table I). ‡ Compared with non-shielded animals of same experiment. § Compared with hypophysectomized, non-irradiated rats (Exp. V). || Compared with non-hypophysectomized rats 4 days after irradiation with 650 r (Table I).
Hyp. = Hypophysectomized. NS = Not significant ($P > .05$).

roughly parallels recovery of globule leucocytes. The extended period of slow recovery, therefore, may be interpreted as being due to scarcity of parent cells from which new globule leucocytes may be formed.

The effect of radiation on globule leucocytes is primarily a direct one as shown by shielding experiments (Table II). Segments of intestine which were either unshielded or paraffin-shielded showed the same reduction in number of globule leucocytes as in irradiated animals not subjected to shielding procedure. In contrast, samples of small intestine which had been lead-shielded during irradiation showed no reduction at 650 r, the LD₂₀ 30 days(5). At 800 r, the LD₁₀₀ 30 days(5), only 40% reduction from normal occurred. X-irradiation may act directly on globule leucocytes themselves or indirectly through changes elicited in their tissue environment. The latter possibility must be considered in view of local disappearance of these cells in areas of tracheal inflammation.

It is probable that radiation-induced stress is responsible for partial disappearance of these cells in lead-shielded segments of intestine of non-hypophysectomized rats receiving

800 r. In the absence of the hypophysis, the comparable LD₁₀₀ 30-day dosage for hypophysectomized rats (600 r) failed to induce a significant reduction in number of globule leucocytes in lead-shielded segments of the intestine (Table II, Exp. V). The shielding procedure was not involved, since in non-irradiated animals, neither shielded nor unshielded segments of intestine showed a statistically significant change in population of globule leucocytes. Neither was it likely to be due to radiations which penetrated the shield, since the theoretical protection afforded by shields was in the neighborhood of 99.5%. Finally, we have demonstrated that treatment with corticotropin or cortisone reduces the number of globule leucocytes(2).

Hypophysectomy, without irradiation, caused a marked reduction in number of globule leucocytes just as it decreases thymic and splenic weight from that of normal rats (6). After x-irradiation of hypophysectomized rat, the number of globule leucocytes was reduced to essentially the same level as in irradiated non-hypophysectomized rat (Table II). The percentage of reduction, however, was significantly less than that of

non-hypophysectomized animals. Hypophysectomy also caused a shift in position of a considerable portion of the globule leucocytes from the tunica propria (where the largest percentage occurs in non-hypophysectomized rat) to the crypt epithelium.

Summary. Within 4 days after whole body x-irradiation (650 r), globule leucocytes were reduced to 1-2% of their normal number in the intestine of the rat. Reestablishment of normal numbers of cells began toward end of the first week after irradiation and was completed by the 5th week. The effect of irradiation on globule leucocytes was chiefly a local one, being prevented by shielding the intestine with lead during irradiation. Only with the LD₁₀₀ 30 days dosage (800 r) was there a significant reduction in number of globule leucocytes in lead-shielded intestinal seg-

ments. This appeared to be the indirect result of stress induced by irradiation, since the effect was prevented by hypophysectomy. Hypophysectomy alone reduced markedly the number of intestinal globule leucocytes.

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Strontium⁸⁵ Metabolism in Man and Effect of Calcium on Strontium Excretion.* (22197)

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(Introduced by Louis Leiter.)

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Sr⁹⁰, a β emitting isotope with a 25 year half life is one of the major hazards of nuclear fission because of its selective localization in the skeleton. Its entry into the human body and its pathways of elimination are, therefore, of great concern. Availability of a carrier-free γ emitting isotope of strontium, Sr⁸⁵, with a half life of 65 days, facilitated this investigation of the metabolism of radiostrontium in man(1). Data on metabolism of intravenously administered Sr⁸⁵ are reported in this communication.[‡]

Methods. Carrier free Sr⁸⁵, 0.1-0.4 μ c/kg body weight was injected intravenously in 4

human subjects. Blood samples and urine were obtained at 1, 4, 8 and 24 hour intervals initially. Subsequently, urine, stool and plasma were analyzed daily. Radioactivity was determined in 1-5 ml plasma and urine and on stool samples weighing up to 5 g. Significant counts on these samples were obtained up to 24 days. A Nuclear scintillation well counter, Model DS3 was used: through special lead shielding the background was reduced to 140-180 CPM.

Results. Fig. 1 shows plasma levels, urinary and fecal excretions of Sr⁸⁵ of a 72 year

* This study was performed under Contract AT(30-1)-1763 between Montefiore Hospital and the U. S. Atomic Energy Commission.

[†] Trainee, National Cancer Institute.

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[‡] Produced in Oak Ridge National Laboratory Cyclotron by the nuclear reaction of Rb⁸⁵ (p,n) Sr⁸⁵. Radionuclides of Sr⁸⁵ were isolated from the target material, Rb₂CO₃, and other impurities with no strontium carrier added. This separation was performed by H. T. Russell, Radioisotope Operations Division of Oak Ridge National Laboratory. We thank these laboratories for their cooperation.

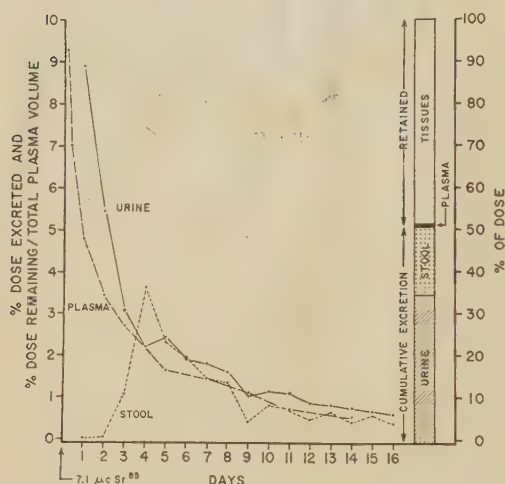


FIG. 1. Intravenous tracer dose of Sr^{85} ; plasma levels, urinary and fecal excretions.

old ambulatory female with osteoporosis. (Case 3, Table I). Initially, plasma levels and urinary excretions declined rapidly, fecal Sr^{85} content was highest on 4th day and from there on plasma levels, fecal and urinary Sr^{85} excretions declined gradually with the lines falling closely together. In 16 days a total of 34.5% was excreted in the urine, 16.3% in the stool; 0.5% was present in the total plasma volume and 48.7% of the dose was retained in the tissues.

Similar experiments were performed on 3 other subjects. Plasma levels and urinary excretions of the first 48 hours of the studies are listed in Table I. Patients 1 and 4 had no

skeletal disease. Patient 2 had Paget's disease of the bone. Significant differences in plasma levels and urinary excretions were noted in the 4 subjects. The 48 hour urinary strontium excretion ranged from 6.5-28.3%. The latter was excreted by pt. 4 who eliminated 57.2% in 15 days in the urine and 11.0% in stool.

After radioactivity of the samples declined to low levels, a second tracer dose of Sr^{85} was injected to each patient at the midpoint of a 2 hour infusion of calcium gluconate, containing approximately 1 g Ca^{++} . The marked enhancement of urinary strontium excretion is shown in column B, Table I. A 5-15 fold increment is noted at one hour. The enhancement continued for several hours after termination of the calcium infusion and was followed by a temporary depression on the next 2 to 3 days. In spite of the marked increase of urinary Sr^{85} excretion, the plasma levels were only slightly different and fecal Sr^{85} excretions were not enhanced. The magnitude of the calcium effect upon urinary strontium excretion becomes evident by comparing the cumulative 24 hour Sr^{85} excretions following administration of the isotope with and without calcium in the 4 subjects: 4.40 vs 21.5%, 4.2 vs 13.7%, 8.9 vs 18.2%, and 18.6 vs 29.5% respectively.

To study whether strontium excretion is similarly enhanced if both the tracer and calcium are injected slowly, the 2 experiments listed

TABLE I. Effect of Intravenous Calcium on the Urinary Excretions and Plasma Levels of Sr^{85} .

Case No.	1 hr		4 hr		8 hr		24 hr		48 hr	
	A	B	A	B	A	B	A	B	A	B
1	.40 (10.49)	6.14 (12.02)	.63 (7.40)	5.82 (7.91)	.76 (6.54)	4.45 (6.61)	2.61 (4.69)	5.13 (4.69)	2.56 (3.48)	1.69 (3.54)
2	.41 (7.37)	4.52 (7.77)	.44 (4.26)	3.70 (4.37)	1.26 (3.42)	2.89 (2.95)	2.05 (2.19)	2.58 (2.27)	2.38 (1.69)	1.19 (1.65)
3	1.17 (12.50)	6.03 (13.62)	.96 (9.26)	4.95 (8.88)	1.26 (7.01)	2.22 (6.93)	5.52 (4.80)	5.01 (4.38)	5.48 (3.43)	3.51 (3.50)
4a	1.38 (10.36)	7.85 (9.01)	3.13 (7.08)	7.19 (5.99)	4.36 (5.08)	6.68 (4.61)	9.80 (3.33)	7.80 (2.80)	9.61 (2.16)	6.16 (2.02)
4b	.09 (1.65)	.11 (1.14)	.87 (5.07)	2.78 (4.85)	5.34 (5.50)	11.00 (5.63)	7.53 (3.60)	9.52 (2.86)	8.95 (2.04)	4.78 (1.65)

Figures represent Sr^{85} in % dose excreted from 0-1 hr, 1-4 hr, 4-8 hr, 8-24 hr, 24-48 hr; % dose of total plasma volume in parentheses.

A = experiments without calcium; B = with calcium, see text.

Exp. 4a and 4b were performed on the same patient; in 4b Sr^{85} was infused in 5 hr without calcium (A) and with calcium (B).

under 4b were carried out. The isotope was infused for 5 hours. The plasma level of Sr^{85} rose gradually and was 5.5% at 8 hours (column A): it declined thereafter at rates comparable to that of the rapid injection of Sr^{85} . Urinary strontium excretion increased gradually and was highest at 8 hours. The cumulative 24 hour urinary excretion was 13.8% (column A). The marked enhancement of strontium excretion by simultaneous but slow administration of the same dose of calcium, approximately 1 g Ca^{++} , infused for 1 hour preceding and for 5 hours simultaneously with Sr^{85} is shown in column B. The cumulative 24 hour urinary strontium excretion rose to 23.4%.

These experiments illustrate that the major route of excretion of intravenously administered strontium is via the kidney; in 15 days as much as 57% was excreted in one patient.

Calcium significantly increases renal clearance of strontium and thereby decreases the body load.

Summary. Radiostrontium metabolism following intravenous administration of tracer doses of strontium⁸⁵ was studied in 4 human subjects. Plasma levels, urinary and fecal strontium excretion were measured. The plasma levels declined rapidly. The main route of excretion is via the kidney. Rate of strontium excretion seems to depend upon the state of bone metabolism. It was low in Paget's disease and high in senile osteoporosis. Simultaneous administration of calcium gluconate significantly enhanced the strontium excretion.

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Mechanical Fragility of Erythrocytes from Patients with Paroxysmal Hemoglobinuria.* (22198)

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It has been established that pH is an important factor in *in vitro* hemolysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH). *In vitro*, hemolysis of PNH erythrocytes in normal human serum is characterized by: 1) a hemolytic system which requires properdin, complement and Mg^{++} (4), all normal constituents of normal serum; 2) absence of abnormal hemolysins or agglutinins in patient's serum(2,3,4); 3) maximum hemolysis at pH 6.5-7.0; 4) minimal hemolysis in normal human serum outside pH range 6-8.

As pH of PNH blood is decreased from pH 7.8, and hemolysis becomes maximum, it is conceivable that the hemolytic system could weaken the erythrocyte cell wall in such manner that *in vivo* trauma would augment

hemolysis. This question was submitted to test by determining mechanical fragility (MF) *in vitro*, of normal and PNH erythrocytes in active and inactive properdin systems at varied levels of pH.

Materials and methods. To determine the effect of decreased pH on MF, hydrochloric acid of N/1.2 to N/8 was used to obtain the desired pH of blood samples. To maintain this pH during rotation, imidazole in HCl (3.44 g imidazole/100 ml of 0.2 N HCl) was used as buffer because of its effectiveness when used in small quantities(7). Although this solution is hypertonic, it becomes approximately isotonic (isotonic equals 1.72 g/100 ml of 0.1 HCl), when it is diluted with equal volume of HCl used to alter pH. Defibrinated blood samples were obtained from 3 normal males, 3 normal females and 3 patients with PNH. Of the 3 PNH pa-

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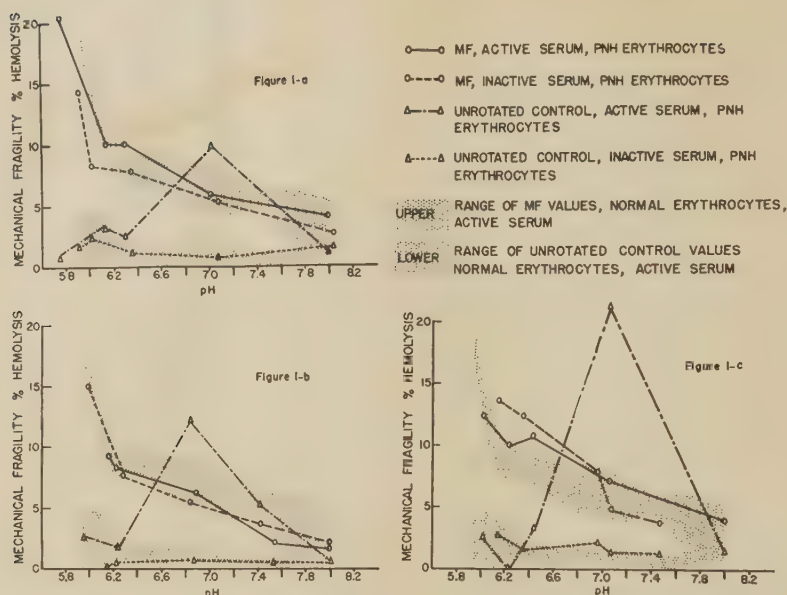


FIG. 1. Results of mechanical fragility studies done at various values of pH on blood of 3 patients with paroxysmal nocturnal hemoglobinuria (Fig. 1-a, 1-b, and 1-c correspond to Cases I, II, and III respectively) and 6 normal subjects.

tients, Cases I and III (Fig. 1-a and 1-c) had approximately 50% transfused cells at time of observation. The erythrocyte population of Case II (Fig. 1-b) contained no transfused cells at time of observation. Each sample was adjusted to 35% hematocrit and divided into aliquots (1.5 ml). The aliquots obtained from each PNH sample were divided into 2 groups, one group was designated the active group and the other inactive. Serum from each aliquot was removed and placed into corresponding tubes. Serums of inactive aliquots were heated in 60°C waterbath for 30 minutes to inactivate complement, thus destroying the activity of PNH hemolytic system, while the hemolytic system of active serum was allowed to remain intact. From the serum of each aliquot, 0.2 of the total volume was removed. This 0.2 volume was replaced by and thoroughly mixed with 0.1 volume of HCl and 0.1 volume of imidazole buffer to obtain the desired pH. The erythrocytes were resuspended in the acidified buffered serum and thoroughly mixed. A pH was determined on each sample in a Beckman pH meter (Model G) before and after the MF determination, the average of the two be-

ing used for interpretation of results. The MF of each aliquot was determined according to the method of Shen, Castle, and Fleming (9) as modified by Ham(1).

Results. Range of values of MF for normal erythrocytes in the pH range 5.95-8.0 is given in Table I and represented by the upper shaded area in Fig. 1. Range of percent hemolysis occurring in unrotated samples of normal erythrocytes subjected to a similar pH range is given in Table I and represented by the lower shaded area in Fig. 1. Normal values represented in Table I and on Fig. 1 were obtained by extrapolation of maximum and minimum values of MF at every 0.2 pH unit from a composite graph containing results of studies on 6 normal subjects.

Results of studies done on three PNH patients are presented in Fig. 1-a, 1-b, and 1-c. The curves labeled unrotated control, active serum, PNH erythrocytes in these Figures demonstrates the hemolysis of PNH erythrocytes in acidified serum. This observation indicates that the properdin system is intact in samples with active (unheated) serum. Correction for hemolysis in the rotated active sample attributable to the PNH mechanism

TABLE I. Range of Values of MF of Normal Erythrocytes at Various Values of pH.

pH	No. of observations	Mechanical fragility		Unrotated control	
		Hemolysis in % Range	Avg	Hemolysis in % Range	Avg
5.95	3	16.8 -19.2	17.5	0 -4.3	1.8
6.0	4	14.0 -15.9	14.6	1.0-5.6	1.9
6.1	4	8.3 -12.2	9.8	0 -4.1	1.4
6.2	6	7.1 -10.4	9.1	.5-2.8	1.2
6.4	6	7.1 - 9.8	8.5	.5-1.8	.9
6.6	6	5.7 - 8.8	7.6	0 -1.5	1.0
6.8	6	4.6 - 8.4	6.5	.6-1.5	1.1
7.0	6	4.3 - 8.2	6.4	.4 - .9	.7
7.2	6	3.8 - 7.0	5.6	.4-1.2	.8
7.4	6	4.6 - 6.0	5.2	0 -1.1	.8
7.6	6	2.1 - 5.9	4.4	0 -1.1	.8
7.8	5	1.6 - 5.6	3.7	.6-1.6	1.0
8.0	4	1.70- 5.0	3.7	.6-1.6	1.3

was done by subtraction of unrotated active control value from the active rotated sample to obtain per-cent hemolysis attributable to mechanical trauma.

The reproductibility of the technic was tested by dividing a sample of normal blood into 7 aliquots and determining the MF of each separately after adding the same amount of acid and buffer. The range of pH values of the 7 aliquots was 6.9 to 7.05 and the range of MF values was 5.2-8.5%.

Morphologic evaluation of cell shape was done by examination of Wright stained erythrocytes. Spherocytes and budded forms were seen at pH 6 in both PNH and normal erythrocytes.

Discussion. It has been postulated that increased MF of erythrocytes may be associated with increased spheroidicity or firm agglutination of red blood cells(10). Increased spheroidicity was observed at pH 6, but no agglutination occurred in the studies. Spheroidicity may be explained as follows: With a decrease in intracellular pH, the amphoteric hemoglobin molecule shifts toward its undissociated state, each hemoglobin molecule releasing a maximum of 10 K⁺. Since each K⁺ acts as an osmotic particle, the osmotic gradient into the cell is increased. This is further augmented by the passage of HCO₃⁻ and Cl⁻ into the cell to neutralize the excess of impermeable K⁺. Osmotic equilibrium is maintained by the passage of H₂O from the

serum into cells thus increasing cell volume (8).

It has been proposed that erythrocytes of congenital spherocytosis, sickle cell anemia and acquired hemolytic anemia may be conditioned in the spleen in such manner as to increase both osmotic and mechanical fragility(5). Perhaps pH is one factor in the "conditioning" process taking place in the spleen. Studies of pH in splenic circulation are not at present available. However, Watson and Paine conclude that red cells in the splenic vein are slightly more spheroidal than cells in the splenic artery(6).

Summary. 1. As pH of blood samples from 6 normal subjects was decreased, susceptibility of normal erythrocytes to mechanical trauma or their mechanical fragility was increased. 2. MF determinations on PNH and normal erythrocytes were done at various values of pH including the range for optimum complement activity in normal serum and it was found that mechanical trauma did not contribute to hemolysis of normal or PNH erythrocytes even though the PNH hemolytic mechanism was active. 3. It is postulated that increased red cell volume due to a shift of Cl⁻, HCO₃⁻ and H₂O into the erythrocyte, may be the mechanism of increasing spheroidicity of the erythrocyte and the MF as a function of decreasing values of pH.

Conclusion. 1. MF of erythrocytes of the PNH patient in an active properdin system does not differ from MF of the same erythrocytes in an inactive properdin-complement system, nor does the MF of these erythrocytes differ from that of normal erythrocytes at values of pH between 8.0 and 5.9. 2. The hemolytic system of PNH, which requires properdin, complement and Mg⁺⁺, does not enhance susceptibility of PNH erythrocyte to hemolysis by mechanical trauma *in vitro*. Hemolysis of PNH and normal erythrocytes attributable to mechanical trauma increases progressively as pH of blood decreases until a critical point (pH 6.0-6.2) where MF rises rapidly.

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Metabolism of Radioactive Para-Aminobenzoic Acid in an *Escherichia coli* Mutant.* (22199)

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While metabolism of para-aminobenzoate (PAB) has been extensively investigated in the mammalian body(1,2), its function and biochemical transformations in microorganisms are still obscure. Conversion of PAB into folic acid and the citrovorum factor occurs in some organisms(3,4) although there is some question whether these are the active forms of PAB. A number of other routes of metabolism have been reported. Davis(5,6) has implicated PAB in the metabolism by *Escherichia coli* of tryptophane, tyrosine and other aromatic compounds, as well as vit. B₁₂. Ratner *et al.*(7) isolated a glutamic acid peptide of PAB from yeast, and Flynn *et al.*(8) have found in 2 strains of streptococci an antibiotic containing PAB and cytosine among its components. Sloane *et al.*(9) identified aniline and p-aminophenol as degradation products in *Mycobacterium smegmatis*. The present studies had their inception in the isolation by Lampen, Roepke, and Jones(10) of an X-ray mutant of *E. coli* which required exogenous PAB for growth but apparently did

not utilize it in producing folic acid. Since the wild strain of this organism normally has no requirement for preformed PAB, it seemed of interest to investigate the fate of this compound labeled with C¹⁴ in such a mutant.

Materials and methods. PAB used was labeled in the carboxyl group with carbon 14 and had a specific activity of 0.037 mC/mg.[‡] The mutant of *E. coli* (ATCC No. 9723a) was grown on basal medium modified from that of Cohen and Arbogast(11) by use of 0.4% glucose. The medium of Lampen *et al.*(10) contained asparagine as well, but this substance was not necessary for maximum growth, and was omitted to facilitate isolations. Organisms were grown in 3 liter batches with aeration for 24 hours at PAB concentration of 0.01 µg/ml. Experiments in which PAB level was 0.05 µg/ml gave similar results. Bacterial suspensions were filtered on Buechner funnel using Celite as filter aid. The filtrate contained less than 20% of total radioactivity. The cells, plus filter-aid, were suspended in water and made up to 60% acetone-water (v/v) by addition of acetone. The residue was filtered off, re-extracted, and extracts, containing approximately half the total cell radioactivity, were combined. Subsequent extractions of cells with hot acetone, chloroform,

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[‡] Purchased from Atomic Energy Commission, Los Alamos Laboratories.

TABLE I. R_f Values of Various PAB Derivatives.

Compound or extract	Solvent 1	Solvent 2
60% acetone extract	.15, .22, .48	.87-.95
PAB	.78	.69
Folic acid		.31, (.19)*
N ¹⁰ -formyl folic acid	.36, .55, .75	.36, .55, .74 (.23, .55, .70)*
Natural citrovorum factor†	.55, .71	.46, .64
Leucovorin	.36, .55, .71	.49, .64, (.65)*
Pteric acid		(.04)*
Pteroylglutamyl-alpha-glutamic acid		(.17)*
Pteroylglutamyl-gamma-glutamic acid		(.35)*
Pteroylglutamyl-gamma-glutamyl-gamma-glutamic acid		(.16, .34)*
p-aminobenzoylglutamic acid	.68	.90
p-aminobenzoylaspartic acid	.62	.88

* These figures from Wieland *et al.*(12).

† Obtained through the courtesy of Dr. K. Schwarz, N. I. H.

and petroleum ether removed practically no radioactivity. An additional 30-40% of the original total radioactivity of cells could be extracted with water. Extracts were lyophilized and kept at -20°C until they were analyzed.

Results. Chromatographic separation of fractions. The bacterial extracts were subjected to ascending paper chromatography on Whatman No. 1 filter paper to investigate the nature of radioactive components. The behavior in the systems investigated led to the conclusion that PAB, folic acid, citrovorum factor, and several other related compounds were not among the bacterial metabolites, although good resolution of radioactive spots was never attained. The R_f values of various substances obtained in 2 systems, including results observed by Wieland *et al.*(12) are indicated in Table I. The solvent systems were 1) methanol, *iso*-butanol, water (1:1:1, v/v) and 2) 5% ammonium citrate buffer of pH 9 saturated with *iso*-amyl alcohol. Folic acid and its derivatives were located by ultraviolet absorption; PAB and its amino acid derivatives by the Bratton-Marshall aromatic amine reaction(13). Several of the folic acid compounds give more than one spot in certain

solvents. Detection of the unknown was solely on the basis of direct radioactive assay of the paper strips, since there was insufficient material present for chemical identification or for spectrum analysis.

Because of the low recovery of radioactivity and poor definition of spots on the paper strips, column chromatographic separations were undertaken. Radioactivity was too tightly bound on Dowex 1 anion exchange resin, but chromatography on Dowex 50 cation exchange resin (10% cross-linkage, 200-400 mesh) provided separation. For elution 0.1 M ammonium acetate buffers were selected because they lent themselves to easy removal. The columns finally used were 100 cm by 0.9 cm, and were initially equilibrated with pH 3.32 buffer. Fractions of about 5 ml were taken and suitable aliquots were radioassayed. Three large radioactive peaks, denoted as B, C, and D in Fig. 1, were detected in the 60% acetone extract. The water extract contained mainly an additional peak, marked as fraction A, along with small amounts of B, C, and D. Fig. 1 shows typical elution patterns obtained at different pH values, PAB has never been seen in more than trace amounts and is eluted in pH 5.9 buffer. The supernatant medium was analyzed in the same chromatographic system and radioactivity was found to consist chiefly of PAB.

For comparison a series of pure compounds were subjected to the same separation procedure. Para-aminobenzoylglutamate and aspartate both emerge at the position of fraction C, but were definitely distinguishable from C

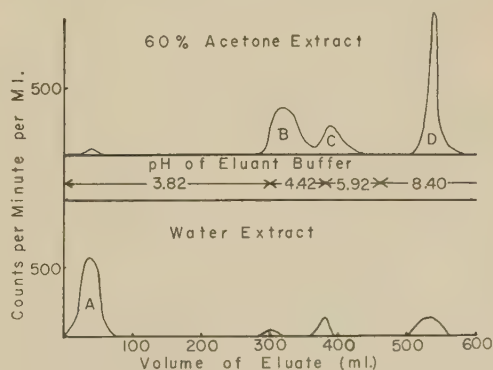


FIG. 1. Elution patterns of PAB metabolites.

by paper chromatography in a solvent of concentrated acetic acid, *iso*-propanol, concentrated ammonium hydroxide (3:2:1, v/v). Fraction C has an R_f of 0.30, while the 2 amides have R_f values of 0.78 and 0.74 respectively. Folic acid, formylfolic acid, and leucovorin are eluted in the general range of fraction D but again have been eliminated as possible metabolites by paper chromatography in the above solvent system, where D has an R_f of 0.30 while the 3 folic acid derivatives have values of 0.55, 0.80, and 0.64 respectively.

Acidic hydrolysates of all 4 metabolic fractions, prepared by refluxing with equal parts of concentrated hydrochloric and formic acids for 16 hours, and the corresponding basic hydrolysates, resulting from boiling with 1 M sodium hydroxide for 2 hours, were rechromatographed on the columns. In every case, PAB was identified as a major component of the hydrolysate.

It seems probable that the 4 metabolic fractions are closely related to each other, perhaps as degradation products, for on rechromatographing each separate fraction, some of the other fractions were formed. During this subsequent treatment fraction D was always formed in largest amount, and may represent the simplest compound.

The addition of methionine, asparagine or a mixture of other amino acids to the growth medium did not alter the elution pattern of the bacterial products, in spite of the observation of Lampen *et al.* (10) that some of these compounds spared a large portion of the PAB requirement of the mutant.

Growth of the mutant with PAB substitutes. A number of other compounds were tested for their ability to promote growth of the mutant. Growth was measured turbidimetrically at 430 m μ . It was felt that extension of such studies, initiated by Lampen *et al.* (10), might facilitate identification of the 4 fractions. Certain of these compounds have been implicated with PAB metabolism in other organisms, either by replacing its requirement for the compound or as metabolites. Some of these data are seen in Table II.

In confirmation of Lampen *et al.* (10), the

TABLE II. Growth Promotion of *E. coli* Mutant by Various Substances.

Drug	Conc. for half max growth—40 hr (μ g/ml)
PAB	.001
p-aminosalicylic acid	.001*
p-aminobenzoylglutamic acid	.1
p-aminobenzoyl aspartic acid	.1
p-formamido benzoic acid	.1
p-formamido salicylic acid	.1
N-formyl-p-aminobenzoylglutamic acid	.1
N-formyl-p-aminobenzoylaspartic acid	.1
Folic acid	1.0
Formylfolic acid	1.0
p-aminohippuric acid	5.0
p-acetamidobenzoic acid	10
p-acetamidosalicylic acid	10
Pantothenic acid	No growth, 5
Aniline	<i>Idem</i> , 5
Vit. B ₁₂	" 5
p-hydroxybenzoic acid	" 5
3-hydroxy-4-aminobenzoic acid	" 5
p-aminophenol	" 5
N-acetyl-p-aminophenol	" 5
Pyridoxine	" 10
Leucovorin	" 1000

* Delayed.

folic acid-like compounds were only weakly effective. Most other substances studied were without effect except for those in which PAB was coupled with an amino acid, or where the amino group was acylated. However since higher concentrations were necessary for comparable growth, it seems likely that these compounds become effective only after hydrolysis. The most active compound, other than PAB, was p-aminosalicylic acid. Delay in growth seen with this substance suggests that it is further metabolized before utilization, and indeed growth in the presence of p-aminosalicylic acid is inhibited by sulfadiazine as if it were PAB. Para-aminosalicylic acid, however, was not eluted from the columns at the position of any of the labeled fractions. The results of Lampen *et al.* (10) with regard to the PAB sparing action by amino acids and purines on growth of the mutant as well as inhibition by sulfadiazine were confirmed. The latter substance inhibits completely at a concentration of 1 μ g/ml in the presence of 0.0005 μ g/ml of PAB whereas 4 other metabolic inhibitors, A-methopterin, desoxypyridoxine, 8-azaguanine and 6-mercaptopurine, were without effect at 10 times this level. The fact that A-methopterin did not inhibit growth

TABLE III. Relative Growth Promotion by PAB Metabolites for 3 Microorganisms.

Compound or metabolite	— $\mu\text{g/ml}$ of medium for one-half maximal growth—		
	<i>E. coli</i> (ATCC 9723a)	<i>S. lactis</i> R. (ATCC 8043)	<i>L. citrovorum</i> (ATCC 8081)
PAB	1	* (100)	* (100)
Folic acid	1000	.2	* (10)
Leucovorin	* (1000)	.2	.1
Fraction A†	3	* (65)	* (140)
B†	* (50)	* (100)	* (200)
C†	* (50)	16	36
D†	5	4	8

* No growth observed at highest concentration tested; concentration given in parentheses.

† Weights of PAB computed from radioactive assay and converted to equivalents of folic acid or leucovorin.

of mutant indicated that folic derivatives might not be closely involved in the anabolic metabolism.

Bacterial growth studies with metabolic fractions. Ability of each of the 4 fractions to support growth of the mutant was then investigated. The possible similarity to folic acid and citrovorum factor was also studied by the ability to promote multiplication of *Streptococcus lactis* and *Leuconostoc citrovorum*, using commercial Difco media (14) for these microbiological assays. Suitable controls were run with mixtures of the appropriate growth factors and the 4 fractions, in order to demonstrate that none of the 4 fractions contained a growth inhibitor. Table III gives the relative concentrations of each fraction for growth of these 3 organisms.

It was possible that the growth stimulation seen in Table III by certain fractions might have resulted from the presence of unlabeled folic acid or leucovorin present as a contaminant. Therefore paper chromatograms of all 4 fractions were prepared under aseptic conditions in 2 solvent systems and the dried sheets were placed on agar plates seeded with *S. lactis* or *L. citrovorum* but devoid of the limiting growth factors. Growth of the organisms was seen only with fractions C and D and was localized at the site of radioactivity. Contaminating folic acid or leucovorin were apparently absent from the fractions, because no growth could be seen where these substances would have been located on the paper strip.

Discussion. While insufficient material has

prevented chemical identification of the metabolites of PAB in this mutant of *E. coli*, the evidence suggests considerable similarity exists between certain of the metabolites and the citrovorum factor. It is of interest that Nichol *et al.* (15) recently reported synthesis from folic acid of various labile precursors and conjugates of citrovorum factor by pigeon liver and *S. faecalis*. Failure to stimulate growth of the mutant by the fractions at concentrations quite comparable to those at which PAB is active might suggest that all fractions are derived from some highly active metabolite degraded as a result of the isolation procedure or that one or more of the fractions consists of a mixture of active and inactive metabolites. The evidence suggests that the substances are quite labile. It remains for further purification and isolation of much larger amounts to elucidate their structure and possible function.

Summary. Radioactive p-aminobenzoic acid has been incubated with an x-ray mutant of *Escherichia coli* which requires this substance as a growth factor. By column chromatography 4 soluble radioactive fractions have been isolated from the cell bodies of the organism, none of them consisting of the original substrate. Two of the fractions possess citrovorum factor activity, but none of them are identical with folic acid, citrovorum factor or certain of its derivatives.

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Effects of Guinea Pig Serum on 6C3HED and AKRL1 Lymphoma Cells Implanted in Various Hosts.* (22200)

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Cells of Lymphoma 6C3HED promptly begin to die when serum from normal guinea pigs is injected into C3H mice in which they are growing(1); yet the lymphoma cells remain viable when they are held in contact with guinea pig serum during several hours at 37°C *in vitro*(1,2). The facts suggest that the animal host somehow participates in the process whereby these lymphoma cells are killed *in vivo*, perhaps by supplying special conditions essential for the process, or by furnishing some substance—an isoantibody, for example, or one or another of the components of complement—that might work with guinea pig serum in producing the effect(1,2). A number of questions related to the facts just mentioned are raised by a more recent observation—namely that cells of Lymphoma AKRL1 continue to proliferate without restraint when normal guinea pig serum is given to AKR mice carrying them(3). Do the AKR mice fail to supply such conditions as might be required for the effectiveness of guinea pig serum *in vivo*, or are they devoid

of some essential host factor? Is there an intrinsic difference in cells of Lymphoma 6C3HED and those of Lymphoma AKRL1, the former being susceptible to the effects of guinea pig serum *in vivo* while the latter are insusceptible? The findings of several experiments, now to be given, bear on these questions.

Results. The experiment of Fig. 1 shows that the cells of Lymphoma 6C3HED were inhibited quite conspicuously when guinea pig serum was given intraperitoneally to AKR mice into which 10 million lymphoma cells had been implanted in each of 2 subcutaneous sites by a technic already described(1), and the finding was confirmed in a subsequent experiment done in precisely the same way. Furthermore, in the experiment of Fig. 1 the inhibitory effect of guinea pig serum was even more marked in the alien and resistant AKR hosts than in the C3H hosts of the sort in which this lymphoma had originated (and which had been implanted with 1 million 6C3HED lymphoma cells in each of 2 subcutaneous situations). This result suggests that the effects of guinea pig serum might depend upon, or be enhanced by, some resistance manifested by the animal

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Inhibition of 6C3HED lymphoma cells in C3H and AKR mice given guinea pig serum

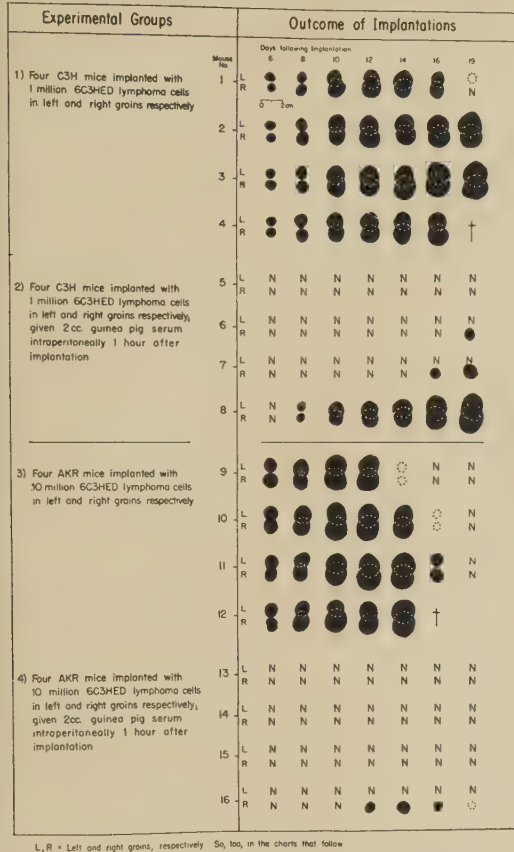


FIG. 1.

hosts, this being the greater in the mice of alien breed. More will be said about this further on.

Next the converse experiment was done. Ten million cells of Lymphoma AKRL1 were implanted in each groin of 8 C3H mice (which were presumably resistant to them since the cells had originated in AKR mice), and 1 million cells were implanted in each groin of 8 AKR mice of the sort in which the lymphoma had originated(3). Four mice of each breed were left untreated as controls and the others were given, approximately 1 hour after implantation, 2 cc of normal guinea pig serum from the same pool as that used in the experiment of Fig. 1. The guinea pig serum had no inhibitory effect whatever on subsequent growth of lymphoma cells in either native or alien hosts; for the cells implanted in

treated and untreated AKR mice grew and formed palpable tumors of approximately equal size, and these all enlarged until death of the animals, as careful chartings showed. Furthermore, the AKRL1 cells that had been implanted in the alien C3H hosts regularly grew to form tumors 10-16 mm across on the 10th day following implantation; but these tumors dwindled and disappeared in 5 of the 8 mice between the 10th and 19th days, while growing progressively in one of the untreated and 2 of the treated animals. The process of regression, although essentially similar in treated and untreated animals, was somewhat more abrupt in the untreated animals, the finding suggesting either that the guinea pig serum might have interfered to some extent with the process of regression or that it might have enhanced growth of these lymphoma cells under these conditions.

In a further test of this sort 2 additional lymphomas of AKR mice — AKR13 and AKR17(3)—were used. Ten million cells of each type were implanted in both groins of 8 C3H mice, 4 of which were left untreated as controls while the others were given 2 cc of guinea pig serum intraperitoneally one hour after the implantations. During the next 6-10 days, tumors measuring up to 17 mm across appeared at every implantation site, the growths in treated animals being precisely like those in untreated controls; within the following 10 days, however, the growths all dwindled and disappeared (Fig. 2), this process too being essentially the same in the treated and untreated groups. In concurrent tests, the guinea pig serum did not influence the outcome of implantations of these cells in AKR mice, the findings confirming observations already published(3).

F1 hybrid C3H x AKR mice were employed in the several further experiments. In the most definitive of these, 500000 6C3HED lymphoma cells were implanted in one groin of 8 hybrid mice, and 500000 AKRL1 lymphoma cells were implanted in the opposite groin; half of the implanted mice were then kept as controls and the others were given normal guinea pig serum intraperitoneally, 2 cc

Tests for effects of guinea pig serum on AKR lymphoma cells implanted in C3H mice

Experimental Groups		Outcome of Implantations						
Group	Mouse No.	Days following Implantation						
		4	8	12	14	16	18	19
1) Four C3H mice implanted with 10 million AKR 1-7 lymphoma cells in left and right groins respectively	1	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	2	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	3	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	4	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
2) Four C3H mice implanted with 10 million AKR 1-3 lymphoma cells in left and right groins respectively, given 2 cc guinea pig serum intraperitoneally	5	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	6	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	7	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	8	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
3) Four C3H mice implanted with 10 million AKR 1-3 lymphoma cells in left and right groins respectively	9	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	10	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	11	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	12	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
4) Four C3H mice implanted with 10 million AKR 1-3 lymphoma cells in left and right groins respectively, given 2 cc guinea pig serum intraperitoneally 1 hour after implantation	13	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	14	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	15	L	●	●	●	N	N	N
		R	●	●	●	N	N	N
	16	L	●	●	●	N	N	N
		R	●	●	●	N	N	N

FIG. 2.

approximately 1 hour after the implantations had been made and 2 cc on each of the following 2 days. Fig. 3 shows the outcome. In the untreated control mice, tumors 10 to 14 mm across were palpable in both groins on the 8th day following the implantations, and these enlarged progressively until death of the hosts between the 18th and 19th days. In the 4 treated mice, tumors similar to those of the control animals appeared in the groins which had been implanted with AKRL1 lymphoma cells, and these growths also enlarged progressively; but the sites implanted with 6C3HED cells remained devoid of tumors. In a further experiment 500000 6C3HED cells were implanted in both groins of 8 hybrid mice and the same number of AKRL1 cells were implanted in each groin of 8 additional animals; half of the mice of each implantation were then given 3 injections of guinea pig serum as in the preceding test. The 6C3HED cells grew as before in the untreated animals, but failed to form palpable tumors in those given

guinea pig serum; the AKRL1 cells formed tumors in the treated and untreated hosts which appeared promptly and grew rapidly and progressively in all instances.

Mention was made above of the possibility that the effects of guinea pig serum *in vivo* might depend upon or be enhanced by some resistance provided by the animal hosts and directed against the lymphoma cells as foreign tissue. If this should prove to be the case, then the effects should be more marked against lymphoma cells that had grown for some days in alien hosts; for under these circumstances such immune responses as the hosts were capable of making should have been stimulated. An experiment was done next to test the possibility. Ten million cells of each of 3 AKR lymphomas previously employed—AKRL1, AKR13, and AKR17—were implanted in both groins of 8 (alien) C3H mice. Nothing further was done to any of the animals until 6 days later, at which time palpable tumors measuring approximately 1 cm across were present at all implanted sites. On the 6th, 7th, and 8th days, 2 cc of normal guinea pig serum were given to 4 of the mice that

Tests for effects of guinea pig serum on the cells of Lymphomas 6C3HED and AKRL1 in hybrid (AKR x C3H) mice

Experimental Groups		Outcome of Implantations						
Group	Mouse No.	Days following Implantation						
		4	8	12	14	16	18	19
1) Four AKR x C3H hybrid mice implanted with 500,000 6C3HED lymphoma cells on left, 500,000 AKRL1 cells on right	1*	L	●	●	●	●	●	†
		R	●	●	●	●	●	†
	2*	L	●	●	●	●	●	†
		R	●	●	●	●	●	†
	3**	L	●	●	●	●	●	●
		R	●	●	●	●	●	●
	4**	L	●	●	●	●	●	●
		R	●	●	●	●	●	●
2) Four AKR x C3H hybrid mice implanted with 500,000 6C3HED lymphoma cells on left, 500,000 AKRL1 cells on right, given 20 cc guinea pig serum one hour after implantation and on each of the two succeeding days	5*	L	N	N	N	N	N	†
		R	N	N	N	N	N	†
	6*	L	N	N	N	N	N	†
		R	N	N	N	N	N	†
	7*	L	N	N	N	N	N	N
		R	N	N	N	N	N	N
	8**	L	N	N	N	N	N	N
		R	N	N	N	N	N	N

* C3H x AKR
** C3H x AKR

FIG. 3.

Tests for effects of guinea pig serum on C3H and AKR lymphoma cells growing in C3H mice

Experimental Groups	Outcome of Implantations											
	Mouse No.		Days following implantation		6	8	10	12	14	16	18	
1) Four C3H mice implanted with 1 million 6C3HED lymphoma cells in left and right groins respectively	1	L	●	●	●	●	●	●	●	●	●	
		R	○	○	○	○	○	○	○	○	○	
			0 2 cm									
	2	L	●	●	●	●	●	●	●	●	+	
		R	○	○	○	○	○	○	○	○	○	
3	L	●	●	●	●	●	●	●	●	●	●	
		R	○	○	○	○	○	○	○	○	○	
	4	L	●	●	●	●	●	●	●	●	●	
		R	○	○	○	○	○	○	○	○	○	
2) Four C3H mice implanted with 1 million 6C3HED lymphoma cells in left and right groins respectively, given 2 cc guinea pig serum intraperitoneally on days 6,7,8	5	L	●	N	N	N	N	N	N	N	N	
		R	○	N	N	N	N	N	N	N	N	
	6	L	●	N	N	N	N	N	N	N	N	
		R	○	N	N	N	N	N	N	N	N	
7	L	●	N	N	N	N	N	N	N	N	N	
		R	○	N	N	N	N	N	N	N	N	
	8	L	●	N	N	N	N	N	N	N	N	
		R	○	N	N	N	N	N	N	N	N	
3) Four C3H mice implanted with 10 million AKR L1 lymphoma cells in left and right groins respectively	9	L	?	●	●	N	N	N	N	N	N	
		R	N	○	○	N	N	N	N	N	N	
	10	L	●	●	●	N	N	N	N	N	N	
		R	○	○	○	N	N	N	N	N	N	
11	L	●	●	●	N	N	N	N	N	N	N	
		R	○	○	○	N	N	N	N	N	N	
	12	L	●	●	●	N	N	N	N	N	N	
		R	○	○	○	N	N	N	N	N	N	
4) Four C3H mice implanted with 10 million AKR L1 lymphoma cells in left and right groins respectively, given 2 cc guinea pig serum intraperitoneally on days 6,7,8	13	L	●	●	●	●	○	○	N	N	N	
		R	○	○	○	○	○	○	N	N	N	
	14	L	●	●	●	●	●	●	N	N	N	
		R	○	○	○	○	○	○	N	N	N	
15	L	●	●	●	●	●	●	●	●	●	●	
		R	○	○	○	○	○	○	○	○	○	
	16	L	●	●	●	●	●	●	●	●	●	
		R	○	○	○	○	○	○	○	○	○	

In similar tests done concurrently, guinea pig serum was given on days 6, 7, 8 to C3H mice that had been implanted with the cells of AKR lymphomas 1-7 and 1-11 respectively. This did not influence the course of events.

FIG. 4.

had been implanted with each type of lymphoma. The outcome in the mice implanted with 6C3HED and AKRL1 cells is shown in Fig. 4. It can be seen that the injected guinea pig serum promptly brought about regression of the 6C3HED lymphomas but had no such effect on the AKRL1 growths. The guinea pig serum given in this way to the C3H mice carrying Lymphomas AKR13 and AKR17 was likewise devoid of effect, though to conserve space the findings are not given in the chart.

The findings just described were extended in several further experiments. In the first of these 6 million cells of lymphoma 6C3HED were implanted in 6 mice of each of 2 alien strains heretofore unused (strains A and DBA), and in 6 C3H mice also; half of the animals were kept untreated as controls while the others received 2 cc of guinea pig serum on the day of implantation and on each of the next 2 days as well. Tumors appeared

promptly in the untreated mice of all 3 breeds and grew progressively to bring about death of the hosts between days 11 and 18, except in one mouse of strain A in which the growths regressed between the 10th and 12th days. Palpable tumors did not appear in any of the mice given guinea pig serum. In a similar experiment, 6 million AKRL1 cells were implanted in both groins of 6 strain A and 6 DBA mice, half of the animals of each breed being treated with guinea pig serum as before. In the strain A mice, palpable tumors 9 to 11 mm across appeared in all treated and untreated animals 7 to 8 days following implantation; the growths in both groups of mice enlarged during the ensuing 4-6 days but thereafter dwindled and disappeared, the process of regression again being essentially the same in treated and untreated animals. In all 3 untreated DBA mice and in one of those given guinea pig serum the AKRL1 cells failed to form palpable tumors, but in 2 of the treated animals they gave rise to bilateral growths that appeared between 7th and 11th days and enlarged progressively until the 20th day when the animals were discarded. A further indication that guinea pig serum can enhance growth of AKRL1 cells *in vivo* was provided by an additional experiment. Eight C57 mice were implanted with 1.5 million AKRL1 lymphoma cells and half of the animals were then given 2 cc of guinea pig serum. Again, the untreated animals failed to develop palpable tumors, but all 3 of those given guinea pig serum developed growths, which measured 12-14 mm across on the 7th day following implantation, enlarged during the ensuing 2 days, and thereafter abruptly disappeared. It remains to be seen whether these findings have a relation to the observations of Kaliss which indicate that certain antisera may enhance growth in alien hosts of transplanted cancers of other types (4,5).

Summary. In the experiments here given, serum from normal guinea pigs acted regularly and powerfully against cells of Lymphoma 6C3HED when these were implanted in mice of various breeds (C3H, AKR, A, DBA, and hybrid C3H x AKR). But it did not inhibit

the cells of Lymphoma AKRL1 in hosts of the above mentioned sorts, and it likewise failed to inhibit growth of cells of 2 additional lymphomas (AKR13 and AKR17) in C3H mice. The findings make it plain that guinea pig serum does not act against the 3 types of AKR lymphoma cells even when these are implanted in alien hosts; indeed in some of the experiments guinea pig serum seemed to enhance the growth of AKRL1 cells in alien hosts. Considered together the observations weigh heavily against the supposition that the mouse host provides natural or induced isoantibodies which act in conjunction with guinea pig serum in killing the cells of Lymphoma 6C3HED *in vivo*, and they sup-

port the inference that cells of Lymphoma 6C3HED differ intrinsically from those of Lymphoma AKRL1, the former being susceptible to the effects of guinea pig serum *in vivo* while the latter are insusceptible.

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Effect of Tumors on Level of Plasma Amino Acids of Eviscerate Rats.* (22201)

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It was expected that the presence of a growing tumor would suppress the rise in plasma amino acids which occurs following evisceration. The contrary is true. The rise is more rapid in eviscerate rats which are hosts to a Walker carcinoma.

Methods. Male rats of the Sprague-Dawley strain were fed Rockland rat diet. Suspensions of tumor were implanted intramuscularly into each thigh near the trunk and permitted to grow for periods of 4 to 14 days. At a weight of approximately 250 g non-fasted rats were anesthetized with cyclopal sodium and functionally eviscerated. Some of the animals were kept without infusions and others were given continuous intravenous infusions of glucose with and without insulin for periods of either 3 or 6 hours. Heparinized blood was collected by cannula from the abdominal aorta and the level of plasma amino

acids determined by the method of Hamilton and Van Slyke(1).

Results. The conditions of individual experiments and the results are shown in Table I. The values are grouped according to the combined weights of tumors of each rat at autopsy. The level of plasma amino acids is not elevated in non-eviscerated tumor hosts. The average values for plasma amino acids increased more rapidly in tumor-bearing rats, the extent of rise showing a positive relationship to the weight of the tumors. Although insulin suppresses the rise in plasma amino acids in all eviscerate animals(2) it does not prevent a more rapid rise in tumor hosts. Whether the extra plasma amino acids arise from the tumor itself, or from normal tissues which are catabolized more rapidly in the presence of a tumor, is not known to us. We have no information about changes in tumor metabolism following evisceration.

Summary. Following evisceration the level of plasma amino acids rises more rapidly in rats which are hosts to a Walker carcinoma

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TABLE I. Level of Plasma Amino Acids in Rats Bearing Tumors. Averages and standard errors.

Glucose load, mg/100 g/rat/hr	Insulin, units/24 hr	Hr	No. rats	Tumor wt, g	Plasma amino acids, mg %
0	0	0	12	36.94 $\pm$ 5.46	3.97 $\pm$.28
0	0	3	12	0.0	9.35 $\pm$.33
0	0	3	12	2.34 $\pm$.39	11.55 $\pm$.45
0	0	3	8	8.98 $\pm$ 1.12	14.26 $\pm$.88
0	0	3	10	22.63 $\pm$.79	15.09 $\pm$.79
0	0	3	10	37.72 $\pm$ 2.73	15.83 $\pm$.60
0	0	6	8	0.0	15.00 $\pm$.82
0	0	6	8	1.68 $\pm$.27	17.91 $\pm$ 1.13
0	0	6	6	8.15 $\pm$ 1.36	20.77 $\pm$.76
0	0	6	6	25.36 $\pm$ 1.50	20.26 $\pm$ 1.13
0	0	6	10	53.78 $\pm$ 6.21	21.64 $\pm$ 1.11
70	0	3	17	0.0	8.82 $\pm$.09
70	0	3	17	19.1 $\pm$ 5.46	10.59 $\pm$.25
70	16	3	17	0.0	4.48 $\pm$.11
70	16	3	17	19.76 $\pm$ 3.41	7.30 $\pm$.44

than in similar rats without tumors. The extent of rise shows a positive relationship to the size of the tumors at autopsy. The differences are evident in the presence and absence of insulin.

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Studies on Agammaglobulinemia VI. Hemostasis in Patients with Agammaglobulinemia.* (22202)

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The discovery of agammaglobulinemia(1) has opened a new field of investigation in immunology and protein metabolism. Appreciable information concerning these patients has accumulated, especially in the field of immunology(2). In all conditions with a congenital or acquired defect of protein synthesis the problem arises whether one is dealing with an isolated error of metabolism or with a group of deficiencies. Typical examples of isolated defects of protein synthesis are the congenital disturbances of coagulation: *e.g.* afibrinogenemia, the various subgroups of hemophilia, hypoprothrombinemia,

etc. Examples of combined defects of protein synthesis are found with severe liver damage, where hypoprothrombinemia is usually associated with deficiencies of prothrombin conversion factors, hypofibrinogenemia and hypoalbuminemia. The association of a number of defects can usually be traced to a dysfunction of the common organ of synthesis.

Because of the existing controversy about the site of synthesis of gamma globulins, it was considered of interest to determine the concentration of the presently known clotting factors and wherever possible to evaluate the adjustment of these factors in response to stressful situations in patients with demonstrated inability to synthesize gamma globulin. Complete tests of hemostasis were performed in 4 children with congenital agamma-

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globulinemic patients. Finally the normal thrombin time excludes any disturbance in the last phase of normal coagulation, *i.e.* in the conversion of fibrinogen to fibrin.

The changes in clotting factors in case F.H. during the course of a fatal hepatitis were in no way different from the changes observed in progressive hepatic failure in a normal individual: the drop in prothrombin concentration from 305 to 66 units/100 ml preceded the fall of fibrinogen from 262 to 110 mg/100 ml and the terminal thrombocytopenia of 72,000 per cumm. The temporary rise of fibrinogen to 449 mg/100 ml in patient J.S. during the course of a febrile episode after the administration of typhoid vaccine is also characteristic of the response observed in normals. Similarly a rise of fibrinogen from 210 to 428 mg/100 ml occurred in patient E.S. during an attack of pneumococcal pneumonia. Upon recovery the fibrinogen level fell to a normal level of 240 mg/100 ml. At no time during the course of these episodes was it possible to detect any gamma globulin in the serum of either patient by electrophoresis. It appears, therefore, that the changes in coagulation factors observed in certain disease states occur in agammaglobulinemia in identical fashion as in normal individuals.

Discussion. The elevation of the platelet count in all 4 cases of congenital agammaglobulinemia and in one adult, who probably had an acquired type of agammaglobulinemia, points toward some functional aberration in the bone marrow. It is of interest in this connection that agammaglobulinemia is characterized by other abnormalities in the hematopoietic system. Among these is the virtual absence from the lymph nodes, spleen and bone marrow, of plasma cells in all cases thus far studied(14). In certain instances absence of eosinophils from the peripheral blood and bone marrow, neutropenia and lymphopenia(3) have reflected the disturbed function of the reticuloendothelial system.

The normal concentration of the plasma clotting factors has several fundamental implications. (1) It suggests that none of the protein factors involved in the coagulation of

blood is a gamma globulin. (2) It indicates that synthesis of all the coagulation moieties produced by the liver is intact in patients with agammaglobulinemia. The liver is the source of fibrinogen, plasma thromboplastin component, prothrombin and the stable and labile prothrombin conversion factors. (3) It supports the concept that the metabolic defect underlying agammaglobulinemia is an isolated deficiency of a single enzyme system. Observations thus far reported(2), including the data submitted here, indicate that synthesis of all proteins, save gamma globulin and the closely related beta 2 globulin is normal in these patients. It also seems of significance that changes in clotting factors during disease states occurred in normal fashion. All these findings are in keeping with previously published data(3) which showed no evidence of abnormality of hepatic function in patients with agammaglobulinemia. These data permit the conclusion that synthesis of gamma globulin and antibody is not directly associated with synthesis of the protein factors involved in hemostasis and support the concept that gamma globulin and antibody are not formed in the parenchymal cells of the liver.

Summary and conclusions. 1. The activity or concentration of the clotting factors recognized at the present time have been determined in 7 patients with agammaglobulinemia. Four of the patients had the congenital-hereditary form of agammaglobulinemia and three had the acquired form of the disease. 2. An elevated platelet count was observed in 5 of the 7 patients. No abnormalities in plasma clotting factors were found. 3. Data presented are consistent with the following conclusions: (a) none of the plasma coagulation factors is a gamma globulin, (b) agammaglobulinemia is probably the result of an isolated deficiency in protein synthesis, (c) the deficiency of gamma globulin synthesis in these patients does not involve the liver but rather depends on an anomaly of protein metabolism existing elsewhere in the reticuloendothelial system.

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Water Intake of Guinea Pigs as Parameter in Oral Drug Therapy of Experimental Irradiation.*† (22203)

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In the study of the effects of drugs on X-ray-induced mortality of experimental animals, the use of peroral route is preferable in many instances to injection because of increased bleeding tendency exhibited by some laboratory animals. This is particularly true with respect to guinea pigs. In this species the pronounced bleeding tendency may cause fatal bleeding at the site of injection. Oral drug administration is usually performed by tube feeding, to control amount of medication as well as possible. This procedure is, however, not suitable for irradiated animals since these are particularly sensitive to infections. The small lacerations caused by such forced feeding experiments may open ports of entry for bacteria and thus superimpose a death rate caused by infection, which would becloud true results of such studies.

Administration of water-soluble drugs with the drinking water appeared, therefore, as a method of choice. Since, obviously, the amount of drug given in this manner would

depend on water consumption of experimental animals, it was necessary to obtain information about their drinking habits.

Methods. Fifty-one male guinea pigs of the Institute stock with an average weight of 450 g (range 250-660 g), were kept in individual cages in air-conditioned quarters. Preliminary investigations had indicated that guinea pigs kept on our standard diet B, consisting of guinea pig pellets, lettuce, and a half orange daily, were drinking quite irregularly. To force experimental animals to drink, lettuce and oranges were withdrawn from the diet. Instead they were offered 235 cc of water to which was added 50 mg vit. C. The water was

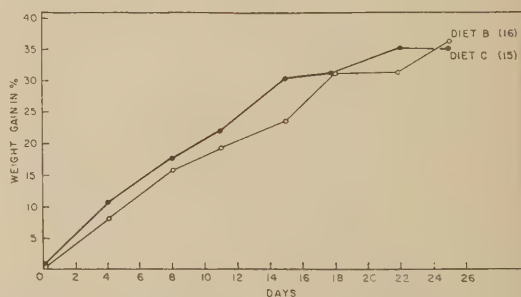


FIG. 1. Comparison between wt gains of guinea pigs kept on standard diet B and experimental diet C (dry food and drinking water plus 50 mg vit. C/235 cc). Figures in parentheses in this and subsequent curves designate No. of animals.

* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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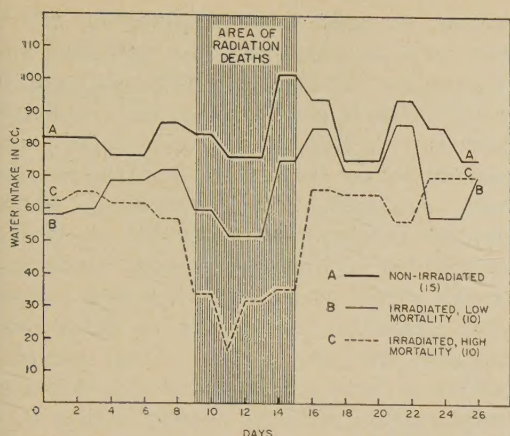


FIG. 2. Analysis of influence of X-ray-induced mortality on daily water intake of guinea pigs. Note decreased water intake in irradiated animals between 9th and 15th postirradiation day is proportional to percentage mortality caused by respective X-ray doses.

given in bottles which were coated with aluminum paint to preclude deterioration of the vitamin in daylight. The amount of water consumed was measured every other or third day.

Results. The first step was to ascertain whether this diet was satisfactory. The weight gain of 16 guinea pigs kept on the usual laboratory diet B was compared with that of 15 animals kept on the new diet C. The result is shown in Fig. 1 which indicates that there was no important difference in weight gain between these 2 groups.

The 15 guinea pigs kept on diet C for 26 days drank an average of 83.6 cc (stand. dev. ± 14 cc). A similar study was then performed on 20 irradiated animals, 10 of which had been exposed to the LD 30/26 days of 2000 KV X-rays and 10 animals which had received LD 80/26 days of 200 KV X-rays. The water consumption in this group averaged 61.7 cc (stand. dev. ± 14.8 cc). Fig. 2 shows in detail the variation in water intake during 26 days' observation. Curve A represents water consumption of non-irradiated animals, curve B that of animals exposed to X-ray dose causing a low mortality, and curve C that of animals exposed to the dose causing a high mortality rate. The time during which death occurred in irradiated animals is marked. There was no important difference

in water consumption between groups B and C until the 9th postirradiation day. At this time some of animals showed symptoms of radiation illness and deaths began to occur in both groups. However, with occurrence of the first fatalities, a notable reduction in water intake in both groups took place, more pronounced in the group exposed to the X-ray dose causing the higher mortality. This observation induced us to consider water intake of survivors separately. In Fig. 3, curve A represents water consumption of non-irradiated animals, curve B shows that of the total group of irradiated animals, and curve C that of all survivors. As this graph indicates, those animals which were to survive, continued to consume water at a fairly constant rate. It is thus clearly recognized that reduction in water consumption was due to the fact that the animals which died reduced water intake shortly before expiration. Some stopped drinking 24 hours before death occurred.

Discussion. Water consumption of irradiated animals during the first week after irradiation was reasonably uniform irrespective of radiation dose. This seems to permit administration of a standard drug dose by mouth with a satisfactory amount of accuracy during this period which is most important for postirradiation protection. The standard procedure of this laboratory, for the evaluation of drugs on lethal effect of total-body

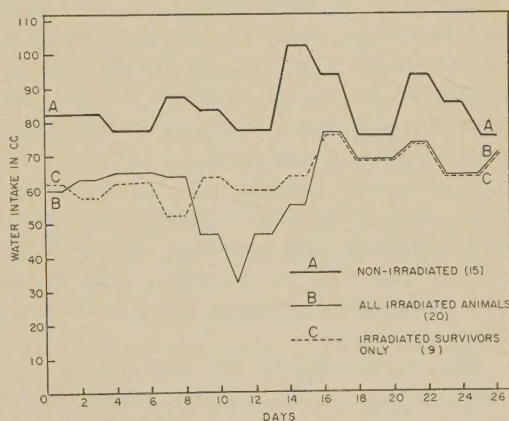


FIG. 3. Comparison of water intake of irradiated animals surviving X-ray irradiation (Curve C) with that of all irradiated animals (Curve B). Note that Curve B does not show the drop in water intake.

radiation, is to administer one fixed drug dose for 5 days after irradiation without adjusting medication to changes in weight of individual animals. The procedure of oral drug administration as outlined appears just as accurate as the injection method.

The oral route offers the advantage of administering larger total drug doses to animals by extending time of medication and of accomplishing this without additional stress to the animals. Extension of injections over equally long periods of time raises the obstacle of burdening the irradiated animal with larger amounts of vehicle substances which may in themselves adversely affect survival rate, as shown by us(1) and others(2) with respect to saline. The saving in time otherwise needed for injections is a further advantage of oral drug administration.

Summary. 1. Drinking habits of guinea pigs kept on a diet of dry food and drinking water have been studied to ascertain the

amount of daily water consumption which may serve as a parameter for oral drug administration. 2. There is a difference between water intake of non-irradiated and irradiated animals. 3. This difference is chiefly accounted for by reduced water intake of animals succumbing to irradiation, which stopped drinking prior to death. 4. Water consumption of irradiated animals is not materially altered during the critical first 8 days after irradiation, irrespective of death rate produced later on. 5. Use of oral administration of water-soluble drugs with drinking water appears therefore possible with a sufficient degree of accuracy.

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A Urinofecal Separator for Laboratory Rat. (22204)

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The problem of obtaining urinary and fecal samples free from intercontamination is a major obstacle to some studies of absorption and metabolism utilizing laboratory animals. In the course of a study of the efficiency of absorption of dietary thiamin by the rat, using a conventional apparatus, serious errors attributable to contamination of fecal samples with urine were encountered. Subsequently a glass separator of new design was constructed which has been shown to effect this separation with a high order of efficiency. The design and operation of this apparatus are described herewith.

The construction of the separator is outlined in Fig. 1. An essential feature is the flared funnel stem which serves to draw the urine away from the pathway of the feces, which fall directly into the feces tube. Glass-to-glass contact must be maintained between

tip of funnel stem and the inside surface of the separator, so that urine may flow readily around the interior of the bulb and be drained off by the urine tube. Urine is prevented from falling directly into the feces tube by the glass cone which rests on three legs $\frac{1}{2}$ in. long. The feces tube is flared to 35 mm O.D. at the top, thereby being extended beneath the shoulder of separator bulb. The wire cage was constructed according to a design obtained from the Nutrition Division, Food and Container Institute, Quartermaster Corps, Chicago, Ill.

The whole apparatus may be attached to a ring stand by means of a 6 in. iron ring to support weight of the funnel and cage, and by 2 clamps fastened to neck of separator and to the feces tube, respectively.

The efficiency of the separator was tested according to the following scheme. Two nor-

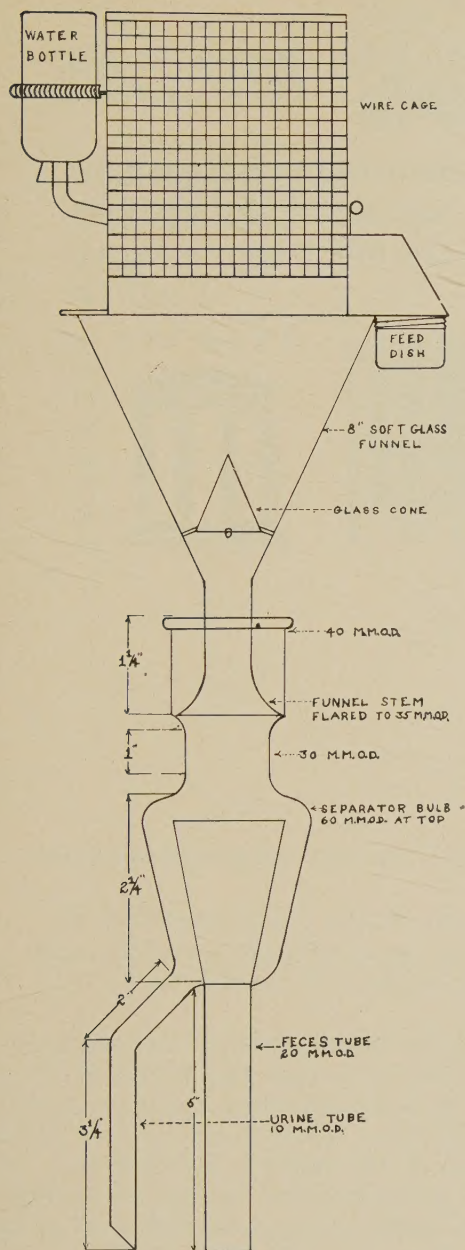


FIG. 1.

had been fasted during the previous 24-hr period, was given an intraperitoneal injection of 120 μg of radioactive thiamin (thiazole-2- C^{14}) having a specific activity of 6 $\mu\text{c}/\text{mg}$. Rat B had been fed a complete diet *ad libitum* during the preliminary period. A collection period of 24 hr ensued, during which time no food was offered. Throughout this interval a composite urine sample from both rats was collected, while the feces sample was derived predominantly from Rat B. Feces excreted by rat B were identified by means of a ferric oxide marker and any pellets excreted by rat A were discarded. At end of collection period the feces from rat B were hydrolyzed using papain and clarase, filtered, and the radioactivity of filtrate was measured by plating, using a Tracerlab windowless flow counter and a Nuclear scaler. Radioactivity of total urine was also determined and efficiency of the system was calculated as percent of urinary radioactivity which appeared in the feces of rat B.

Based upon 12 such tests, the average radioactivity in the feces, expressed as a fraction of total urinary radioactivity, was 0.11% with a standard deviation of 0.08%. No contamination of urine by feces was evident from visual examination.

The performance achieved by the apparatus described is sufficient for most research purposes. By appropriate modifications in scale, the separator may be adapted to smaller or larger laboratory animals than the growing rat. By introducing ground glass fittings and a glass cage, the apparatus also has been converted to a closed system for the simultaneous collection of respiratory gases. Some increase in ease of handling and in rigidity has been attained by the use of a pyrex funnel with the stem fused directly to the neck of the separator.

mal rats, A and B, weighing about 100 g, were placed in the wire cage. Rat A, which

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ERRATUM

Oct. 1955, Article 21941, by Greenway and Quastel, Column heading, Table I should read " $\mu\text{l CO}_2$ above endogenous evolved in 1 hr".
P. 73, col. 2, line 33 "0.25 M" should read "0.025 M."

